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SCANNING ELECTRON MICROSCOPY OF LAMINA PROPRIA AND SUBMUCOSA OF COLON

by

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A THESIS

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ABSTRACT

In modern surgery, the incorporation of the submucosal layer of the bowel wall is considered to be essential for a sound surgical bowel anastomosis. In pathology, the configuration and changes in the lamina propria and submucosal layer of the intestinal tract are frequently crucial for proper diagnosis of a disease.[26,33,35,77] The structure and, to a degree, the function of the lamina propria and submucosa, in the gastrointestinal tract, have been described using light microscopy.[5,47,76] Using two-dimensional tools, such as light microscopy and electron microscopy, the anatomists and pathologists have described normal and diseased lamina propria and submucosa. Over the last 20 years, attention has been paid to the detailed ultrastructure of the mucosal epithelium.[3,7,9,39,41,56,87] Submicroscopic structure of the bowel wall, innervation and the structural characteristics of the muscular coat have also been documented.[36,46,62,74] However, description of the architectural arrangement and fine structural detail of the lamina propria and submucosa of the gastrointestinal tract remains sketchy. Recently a small number of investigators have provided additional information on the structure and components of the above-mentioned layers.[18,50,69] The majority of this work was done using the transmission electron microscope (TEM).[20] It is conceivable that the histological features and, possibly, changes occurring in the lamina propria and submucosa may be easier to be recognized and diagnosed in a three-dimensional tissue preparation. During 1976-77, Lord and associates initiated attempts to gain topographic structural information on the three dimensional arrangement and ultrastructure of the surfaces of intestinal lamina propria.[50] They used the scanning electron

microscope (SEM) as the principle tool of investigation.

The object of this work was to refine and perfect existing methods, and to devise new methods of investigation to clearly describe the pattern and ultrastructure of the lamina propria and submucosa in the colonic tissue of rat, dog, and man. The principle tools of investigation were the scanning electron microscope (SEM), the stereoscopic light microscope (SLM), and routine histology using Hemotoxylin and Eosin stain. A description of the normal colonic features and ultrastructure in the three types of animals has been selected prior to observation of the changes that take place in a disease process involving colonic tissues. Successful development of relatively simple techniques made it possible to remove the epithelial cell elements of colonic mucosa from the surfaces of lamina propria and submucosa. The prepared samples were subjected to a modified freeze-drying process and were treated with gold for scanning electron microscopy. Portions of each specimen were examined under light microscopy and scanning light microscopy. It was found that the lamina propria possesses a pattern of pits. These were recorded by photography and characterized by description and measurements. The arrangement of the submucosal collagen fibres was, as expected, very random. It was studied from the aspect of layering and fibre bundle size. The differences and similarities of the studied layers were documented in all three species -- rat, dog, man.

JUSTIFICATION OF THE PRESENTED WORK

A systematic ultrastructural investigation of the intestinal wall, submucosa, and lamina propria has not been carried out. The present knowledge of these two layers consists of classical histological descriptions[5,48,76] and incidental descriptions using transmission electron microscopy when other layers of bowel wall have been studied.[20,46,69,82] Encouraged by the initial results of Lord et al,[50] methodical observation of the two layers, lamina propria and submucosa, was initiated using scanning electron microscopy as the tool of investigation. The existing potential of a relatively new tool, for diagnostic purposes in bowel pathology, requires development of appropriate techniques and correlation of the known structural facts with newly acquired images of the normal tissues. It was necessary to learn as much as possible about the normal structure of the healthy lamina propria and submucosa, as seen under the scanning electron microscopy. Relatively new techniques and improved methods had to be tested and perfected before the new technique could be applied to interpretation of changes in the diseased segments of bowel wall.

The three-dimensional ultrastructure of the lamina propria and muscularis mucosae and submucosa of colon had to be ascertained, verified and described before the effects of disease known to involve these layers could be thoroughly examined in three dimensions.

This thesis will serve as an essential stepping stone for further work in investigation of pathological changes involving the ultrastructure of the diseased colonic wall.

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C H A P T E R I

INTRODUCTION

INTRODUCTION

In the past, little attention has been paid to the structure of the lamina propria and submucosa of the wall of the colon. The functional implications of the submucosa and lamina propria, as regards to strength, embedding of blood vessels, nerves, and lymphatics, as well as glandular components, has been neglected.

The submucosa is largely responsible for the physical strength of the intestinal tract wall.[84] Despite its importance, little is known about the inner structure of this layer. The inert chemical nature and inherent strength of the collagen fibre makes collagen probably the major barrier preventing bowel wall perforation.[70] Until now the description of the submucosa was a by-product from a study of Meisner's plexus, which is present in the substance of the submucosa.[36, 62] Biochemical studies of the properties and features of mammalian collagen has produced a massive body of information.[70] Most research is being carried out on collagen of the skin, joints and assorted connective tissues. To our knowledge, no definitive study has been published describing the characteristics of intestinal wall collagen.

The contribution of the lamina propria to the strength of the bowel wall has not been determined or documented. Functionally, the lamina propria provides a template for the surface relief of the mucosal lining of the bowel. However, the reverse statement may also be true. Lamina propria serves as a substrate for anchoring the columnar epithelium and allows the blood supply and lymphatics to reach the surface of the intestinal mucosa.

Investigation of the ultrastructure of the bowel wall by other

workers included the micro-detail study of the cells of the mucosal epithelium.[9,39,41,68] Innervation and ultrastructure of the intestinal muscular coat has been documented.[4,46,68,74] The majority of studies reviewed for the preparation of this thesis were carried out under transmission electron microscopy (TEM). Only recently, studies appeared utilizing scanning electron microscopy (SEM).[1,2,18,32,42,56]

An exhaustive research of the literature produced no significant accounts of the microstructure of the lamina propria or the submucosa of the mammalian gastrointestinal tract, except for the work done by Lord et al.[50]

As a principle, it is necessary to investigate the potential advantages and disadvantages of a new tool, such as the scanning electron microscope, for it may provide a new dimension to the diagnostic capabilities of modern medicine. Under two-dimensional light microscopy and transmission electron microscopy, changes in lamina propria such as irregularity, loss of normal definition, flattening of the mucosal relief, trabeculation, fenestration and other minute changes have been documented.[61,77,91] With the use of a three-dimensional tool, such as SEM, changes of minor magnitude may be observed, making the diagnostic process more sensitive and pathological interpretation more precise. Three-dimensional SEM provides more information on the observed surfaces and it is faster than the traditional two-dimensional modes of observation. Using the new tool to full advantage, the interrelation between the observed surfaces and the structural components on and immediately beneath the surface may be observed and described. In conjunction with standard microscopic techniques, the use of SEM may be developed into a

valuable diagnostic tool for pathology.

It is proposed to further study the early and advanced pathological changes taking place in the diseased colon with special emphasis on Crohn's disease. The present work developed the techniques necessary for the observation of such changes in three-dimensional terms of reference.

This thesis will describe the normal features and characteristics of healthy colonic lamina propria and submucosa in the three animal species, rat, dog, and man. Sufficient information regarding the normal appearance and ultrastructure of the lamina propria and submucosa serves as a stepping stone to investigation of pathological changes at the ultrastructural level of the diseased bowel wall of the colon and, in the future, of any other portion of the gastrointestinal tract.

A. Brief Review of Colonic Wall Histology

The distal portion of the intestinal tract, the colon, is grossly subdivided into six confluent parts: 1) caecum, 2) ascending colon, 3) transverse colon, 4) descending colon, 5) sigmoid colon, and 6) rectum. These portions of the colon have basically the same microscopic structural arrangement: 1) mucosal epithelium, 2) lamina propria, 3) muscularis mucosae, 4) submucosa, 5) tunica muscularis (two coats), and 6) serosal coverings.

1) Mucosal Epithelium

The mucosal epithelium of the colon consists of a simple columnar epithelial layer. The absorptive and mucous-secreting cells have an ovoid or round nucleus located at the base of the columnar epithelium.[5,47] At the free luminal surface border, microvilli can be observed.[76] The terminal bars, the majority of the mitochondrial content, the endoplasmic reticulum and the Golgi apparatus are located in the upper portion of these columnar cells.[9,39,54] From the flat colonic surface, which lacks the villi, the Crypts of Lieberkühn drop directly into the substance of the lamina propria. The surface of the lamina propria and the crypts are lined with the strong basement membrane. The lining of the crypts is composed mainly of mucous cells, a small number of columnar epithelial cells, and few undifferentiated cells.[47]

2) Lamina Propria

The lamina propria is a substrate layer for the epithelial coverings of the mucosa. It is composed of loose reticular connective tissue, containing a small number of arterioles, venules, the capillary bed and the lymphatics.[47] The lamina propria is indented by the densely

packed Crypts of Lieberkühn. Within the lamina propria, multiple lymphatic nodules of various sizes are embedded. The lamina propria is made of fibroblasts and other cellular elements.[20] Arterioles, venules, capillaries and lymphatics of the colon do not appear to have any definite orderly arrangement.[47]

3) Muscularis Mucosae

This is a very thin layer of smooth muscle which may be present between the lamina propria and submucosa. It consists of an inner circular layer and an outer layer of longitudinally-oriented muscle fibres, and binds and connects the lamina propria and submucosa firmly together. It also allows for a degree of independent movement of the two layers,[47] and provides motility for lamina propria.[47]

4) Submucosa

The submucosa is a relatively homogenous layer of the intestinal wall, composed of multi-directionally arranged collagen fibers and bundles, embedded in the ground substance. Within the submucosa, an ill-defined plexus of blood vessels and lymphatics is located.[47] Heller's and Meisner's plexuses are embedded in the submucosa along with the other structures.[47] An occasional lymphatic nodule penetrates the lamina propria indenting the submucosa. There is no orderly arrangement in this layer, it is loosely attached to the adjacent tunica muscularis.

5) Tunica Muscularis

The tunica muscularis is composed of two layers of smooth muscle. The circularly arranged inner layer and the longitudinally-oriented outer smooth muscle layer are perforated by perpendicularly travelling blood vessels. Between these two layers, Auerbach's plexus is found.[30]

6) Serosa

The serosa is the outer-most layer of the colonic wall coverings. It consists of an inner layer, containing the bulk of the connective tissue, and of an outer layer, the surface of the mesothelium. The serosa envelopes the longitudinally travelling blood vessels and the frequent fat lobules on the outer surface of the colonic wall. Histologically, the structure of the colonic wall in the three species presented is essentially the same.

A histological arrangement is schematically outlined in Figure 1.

B. Blood Supply of the Colon

The subserosal superficial arteries and veins of the colon run along the outer surface of the serosa to the point where they branch and pierce perpendicularly the two layers of the muscular coat. A minimal amount of intercommunication between arterioles and venules is noted in the muscularis and submucosal interface. As the vessels enter the submucosa, dense vascular plexuses are formed. From these plexuses, arterioles and venules of a very small caliber continue towards the mucosa. These perforate through the remainder of the submucosa, muscularis mucosae and enter the substrate of the lamina propria. The arterioles and venules then supply a profuse network of mucosal cappillaries, the connective tissue of lamina propria supports lymphatic draining channels. The arrangement of the vessels and lymphatics in the colonic wall is relatively disorganized.[47,58]

A diagrammatic illustration of the distribution of the blood supply of the colonic wall is presented in Figure 2.

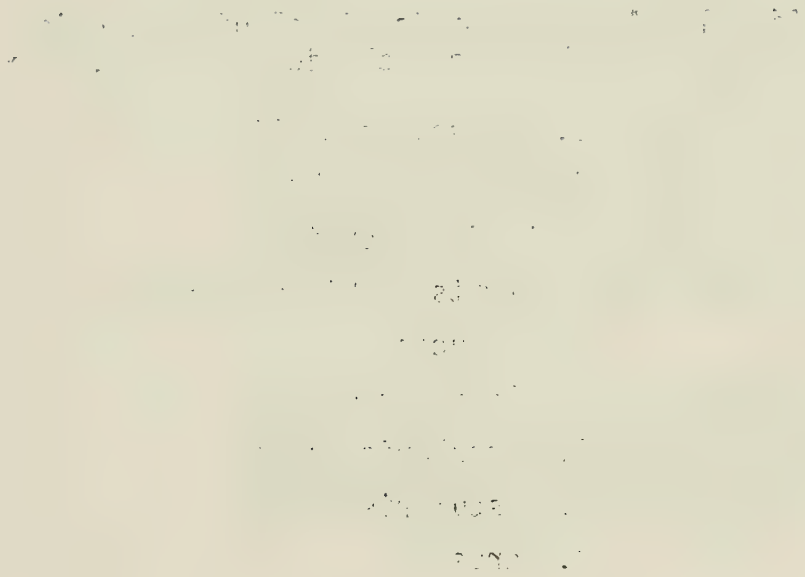
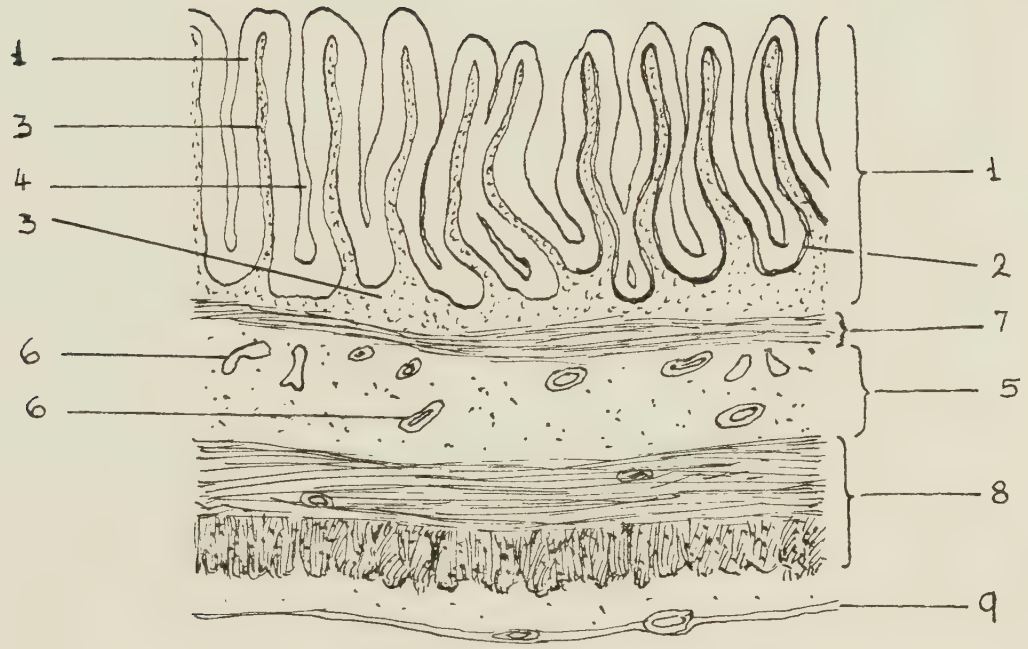


Fig. 1. A diagrammatic histological section of the human colonic wall. Mag. 300x.

1. Mucosal epithelium
2. Basement membrane
3. Lamina propria
4. Crypts of Lieberkühn
5. Submucosa
6. Blood vessels
7. Muscularis mucosae
8. Muscularis
9. Serosa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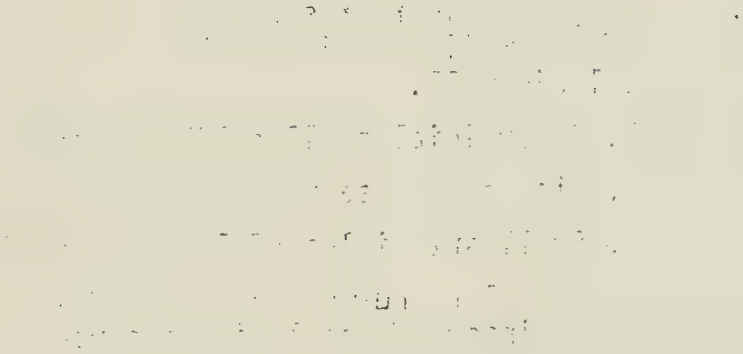


Fig. 2. Diagrammatic representation of the blood supply of the colonic wall.

1. Superficial small caliber vessel
2. Rich submucosa vascular plexus
3. Small arterioles of lamina propria
4. Capillary network and lymphatic drainage of the lamina propria

4
3
2
1

C. Literature Review of SEM Studies of Lamina Propria and Submucosa

A study of the lamina propria and submucosa of the bowel wall under SEM was carried out at the University of Alberta. To the best of our knowledge, there has been no published work on the topic except for the work of Lord et al.[50] Lord's study was concerned with the healing process of the connective tissue of the submucosal layer of the bowel. It documented the surface structure of the lamina propria and muscularis mucosae. Initial techniques for the present study were derived from Lord's work.[50] Later they were modified and improved.

D. Lamina Propria and Submucosa Histology Under Transmission Electron Microscopy (TEM)

The work that has been done on the structure of the lamina propria and the fibre arrangement of the submucosa of the intestinal tract, has been done exclusively with the transmission electron microscope. It has demonstrated the irregular fibre pattern of the submucosa and the presence of fibroblasts and macrophages in the fibrillar network of the lamina propria.[69] Only passing references have been made regarding the structure of the submucosa and the lamina propria as randomly arranged collagen fibres, embedding vessels, lymphatics and other structures in the region.[20,43,44,69] Thus, there was scanty information available for comparison and correlation with new images acquired by the use of SEM.

E. Scanning Electron Microscopy -- Basics of the Technique

The process of utilizing the scanning electron microscope begins by coating a dehydrated specimen with approximately one hundred Å of gold. The coated sample is placed into the microscope vacuum chamber.

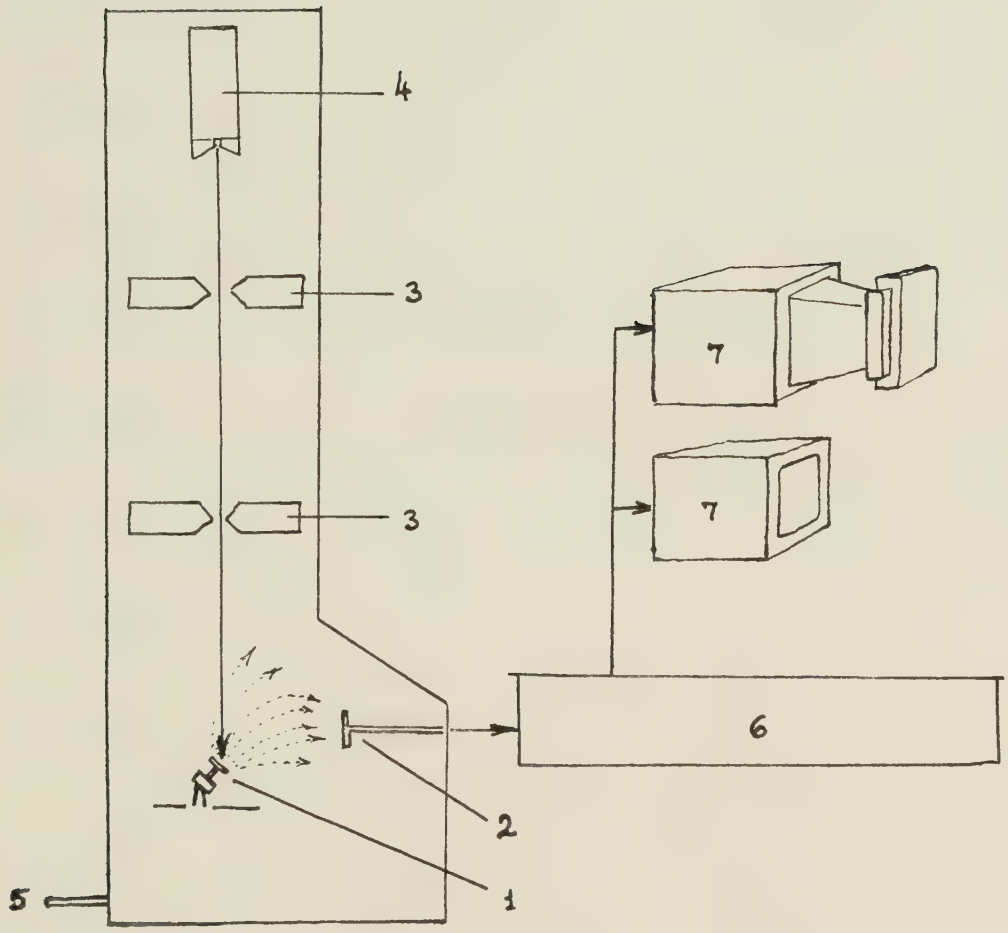
The image of the observed surface is obtained by bombarding the surface layer of the specimen systematically with a 70 Å wide beam of electrons. These electrons, as they strike the surface of the sample, generate a cloud of secondary electrons which are in turn attracted to a collecting plate. The variable electrical potentials from the collecting plate are processed and translated electronically into a meaningful picture that can be observed and photographed on the screen. (Fig. 3)

F. Principles of Freeze-Drying

Use of SEM requires that specimens be devoid of water or of any other volatile substances that may contaminate the chamber or prevent the operation of the scanning electron microscope. One method of dehydration is freeze-drying. Freeze-drying removes the water by converting fluid H_2O into a solid. The extremely rapid temperature drop prevents excessive ice crystal formation. Thus, it prevents damage to tissues. By maintaining a relatively high vacuum and a relatively low temperature, the ice sublimates from the tissue. If used properly, the technique produces minimal damage to the tissue.

Fig. 3. A diagrammatic representation of the scanning electron microscope (SEM).

1. The sample on the stub
2. The collector plate
3. Magnetic lenses
4. The electron gun
5. The vacuum system
6. The electronics
7. The screen and photo apparatus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CHAPTER II

MATERIALS AND METHODS

METHODS AND MATERIALS

A. Materials

For the purposes of this study, colonic tissues from three species were selected; 1) rat, 2) dog, and 3) man. The first two species were selected due to ready availability and histological similarity to the human colon structure. Human tissue was used to determine the normal appearance of the human colonic lamina propria and submucosa as, in the future, the work will primarily involve the study and analysis of changes in human pathologic specimens.

1. Rat

Young, healthy female Wistar rats weighing between 250-300 grams were used. They had been maintained on a regular, staple diet and then starved for twelve hours prior to the procedures.

2. Dog

Young, healthy mongrel dogs of both sexes were utilized for this study. A mid-colonic portion of bowel was resected and the colon perfused.

3. Man

Relatively normal human colonic samples were obtained from the hospital operating room. Normal portions of the colon were obtained from the surgically resected diseased bowel. The age of the patient and the disease of the patient have been considered prior to selection of the specimen. Tissue samples have originated from the proximal end of the surgically resected mid colon.

B. Number of Specimens

Due to the relatively uniform nature of the submucosa and lamina propria, six valid specimens from each category will be presented. There will be six categories; rat, dog and man lamina propria, and rat, dog and man submucosa. It must be mentioned here that the number of specimens examined during the course of this study was much greater than the number finally selected for this presentation. This study will limit itself to examination of the "typical" appearance of colonic lamina propria and submucosa in man and the two experimental animals. It also should be mentioned that the gastric and rectal regions of humans were sampled and studied. In the experimental animals, the gastric, duodenal, jejunal, ileal, caecal, colonic and rectal samples were prepared and investigated. However, these will not be reported or described in the course of this study.

C. Specimen Collection

In the experimental animals, a 3-10 cm portion of the midsegment of the transverse colon was resected just before euthanasia of the animal. In humans, the site of the collection of the specimen was determined mainly by the location of the pathology and the length and site of the resected bowel. The most frequent site of resection of the bowel was the proximal part of the descending colon. In all instances, the position of the tissue sample obtained was as close to the mid colon as possible. As soon as the specimen had been collected, whether in the experimental animal or humans, it was rushed to the laboratory for initial treatment of the surfaces of the lamina propria and submucosa.

D. Methods of Investigation

Having obtained the acceptable segment of the colon from rat, dog, or man, the specimen was cleansed. A portion of the specimen was then fixed for histology. The remaining portion of the specimen was surface treated in order to remove the mucosa. This was done under direct control of scanning light microscopy. The specimens were fixed and mounted on a steel SEM stub. Dehydration and gold coating preceded observation of the specimen under scanning electron microscopy. Results of histology, scanning light microscopy and scanning electron microscopy were documented and analysed by color or black and white photography. Diagrammatic representation of this mode of investigation is found in Figure 4.

The techniques of Lord et al[50] were used for this study. They consist of; 1) collection of the specimen, 2) cleansing the specimen mechanically by a scalpel, 3) cleansing the specimen using hyaluronidase, 4) treatment of the specimen with glutaraldehyde, 5) treatment of the specimen with osmium tetraoxide and chemical dehydration through increasing concentration of alcohols and amylacetate. The specimens cleansed in amylacetate were critically dried and coated gold.[50] Very soon it was realized that this technique had serious problems. For example, 1) it required a minimum of 14-16 hours to complete the entire process of treatment and dehydration of the samples, 2) a need for critical point drying, 3) the human handling of potentially dangerous substances, such as osmium tetraoxide, amylacetate and explosive aromatic compounds, 4) frequent and heavy contamination of the samples with dust particles and crystals from the solutions used for fixation and dehydration, 5) only a very small number of samples could be

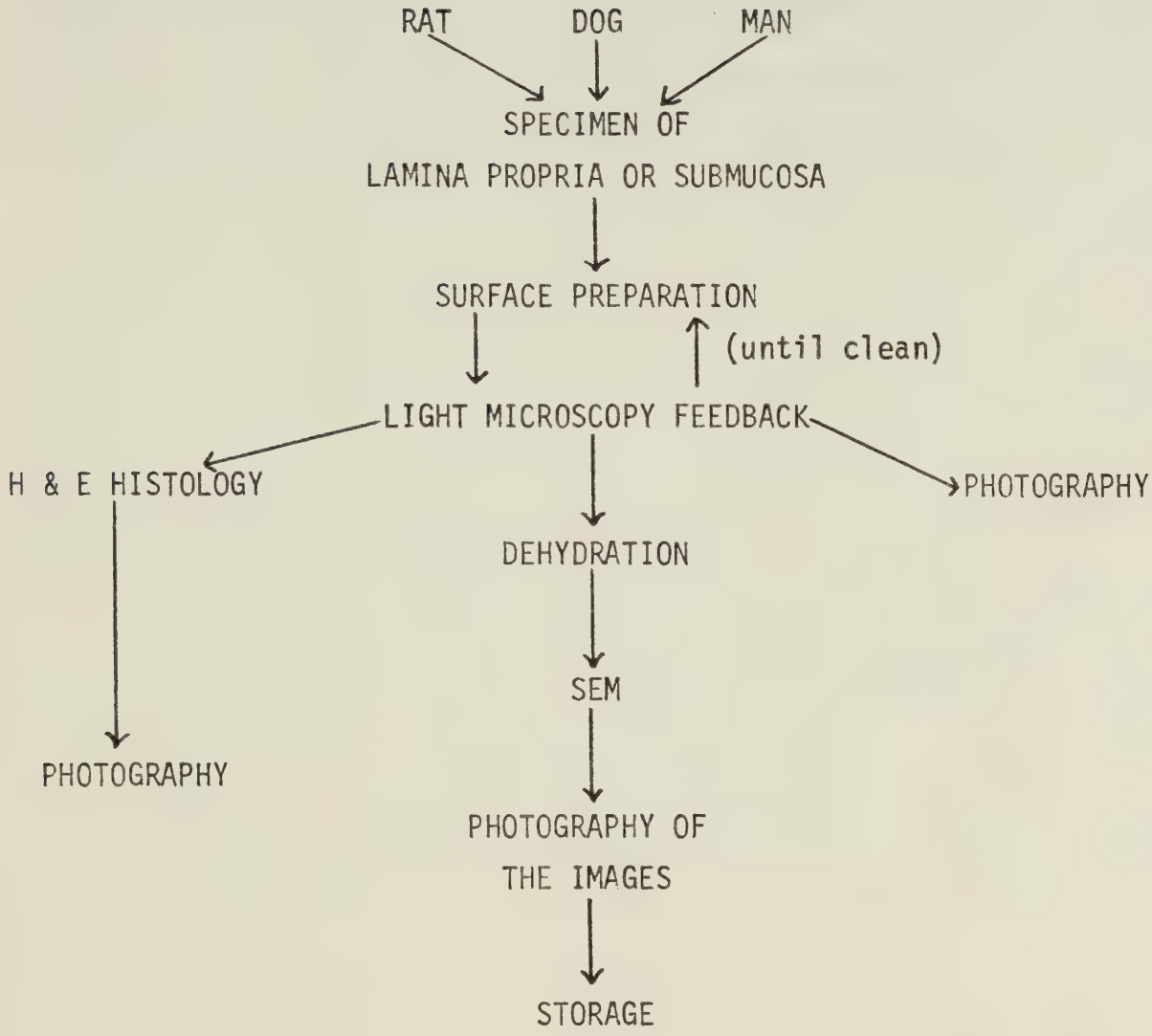

RAT
DOG
MAN
SPECIMEN OF
LAMINA PROPRIA OR SUBMUCOSA
SURFACE PREPARATION
(until clean)
LIGHT MICROSCOPY FEEDBACK
H & E HISTOLOGY
PHOTOGRAPHY
DEHYDRATION
SEM
PHOTOGRAPHY
PHOTOGRAPHY OF
THE IMAGES
STORAGE

FIG. 4.

processed in 24 hours, 6) a distortion of the sample surfaces during the chemical drying, 7) there was no feedback mechanism to ascertain if the sample has been sufficiently abrased and cleaned by the scalpel blade before the sample was fixed and dryed.

C H A P T E R III

SURFACE PREPARATION TECHNIQUE

SURFACE PREPARATION TECHNIQUE

A. Development and Improvement of the Surface Preparation Technique

The initial techniques employed in preparing this study required overhauling. As for the surface preparation technique, it was necessary to make it as simple as possible, fast and highly reproducible. The technique must not introduce any measurable artifact; it should be non-contaminating to tissue and non-hazardous to both handlers and tissues. Most importantly, the technique should incorporate a type of a feedback system to determine whether or not the surface had been exposed to the necessary extent (Figures 5 and 6).

The introduction of routine use of scanning light microscopy produced remarkable improvements in the surface quality of the specimens. This is demonstrated in Figure 7. Insufficient or excessive removal of tissue obscuring the desired surface was minimized using scanning light microscopy. The amount of debris left on the observed surfaces decreased significantly as demonstrated in Figure 8. The technique of using a #15 scalpel blade for abrasion and removal of epithelial layer of mucosa worked relatively well on the intestines of rat. However, the technique left a lot to be desired when used on the colon of the dog and human specimens. The relatively thick mucosal epithelial cell lining and the depth of the crypts posed a serious problem for the simple scalpel abrasion technique. The main problem appeared to be in removal of large portions of epithelium along with underlying lamina propria. Thus, very poor quality micrographs were obtained at first. Attempts were made to improve this by use of straight blades, scalpel, use of ultrasound, high speed waterjet, compressed air, acetic acid,

and exposure to hyaluronidase. The above-mentioned techniques were ineffective in decreasing the amount of debris left on the surfaces and in decreasing the amount of damage sustained by the lamina propria or submucosa. Accidentally it was realized that if one crushes the epithelial layer, one will sheer off most of the epithelial lining of lamina propria without damaging the lamina propria. Sheered off and crushed cells and debris were forced out of the Crypts of Lieberkühn in the cleansing process. If a finger is used as the crushing instrument, the damage incurred by the structure of lamina propria is minimal. Straight scalpel #11 was always used with the aid of SLM.

The introduction of this simple modification of Lord's technique improved the ease and quality of the surface-abrasion technique. Figure 9 demonstrates the extent of the improvement.

Experimentation proved that immersing the unprepared specimen into 2% formalin for 10-15 minutes makes the epithelium easier to remove and causes, in turn, less debris to be left on the surface. This short exposure to formalin, for some reason, enhances the removal of the thick muscularis coat away from the submucosa. The removal of the convoluted muscularis layer allows for a much more stable and smoother surface of lamina propria or submucosa and permits better removal of mucosal surface.

B. Surface Cleansing Technique

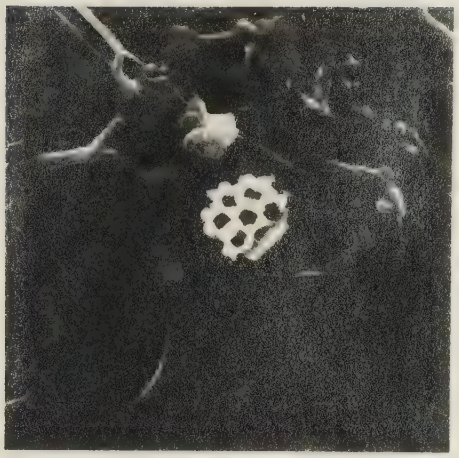
1. The intestinal specimen was cooled to room temperature by emersion in saline at 4°C.

2. The specimen was cleansed with running water, the lumen was opened at the mesenteric border and the extraluminal tissue was trimmed.

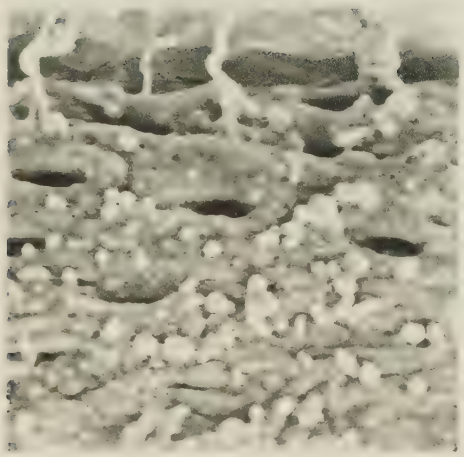
Fig. 5. Contamination of the observed surface by:

A. and B. Solution crystals,
C. Air contaminants, eg. dust
D. Specimen deformation during chemical dehydration.

A



B



C

D

Fig. 6. Lack of feedback mechanism for surface cleansing procedure.

A. Surface insufficiently scraped

B. Surface too severely scraped

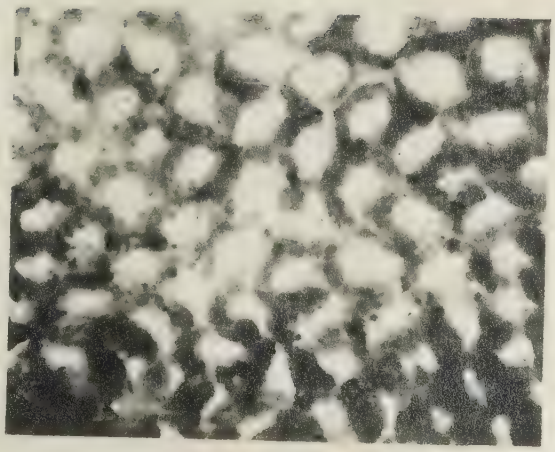
C. Surface sufficiently scraped, but littered by debris

Fig. 7. Two photographs of dog lamina propria taken via light microscope.

A. Insufficiently scraped sample

B. Minimal debris present

A



B

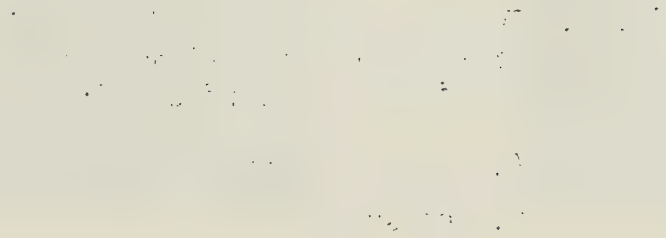
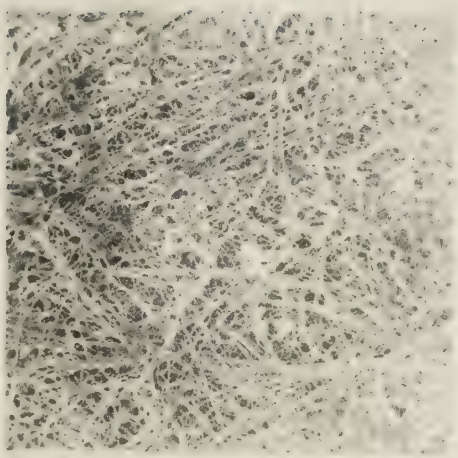


Fig. 8. Two SEM micrographs documenting presence of minimal amount of debris on the surface of:

A. Dog lamina propria

B. Dog submucosa

A



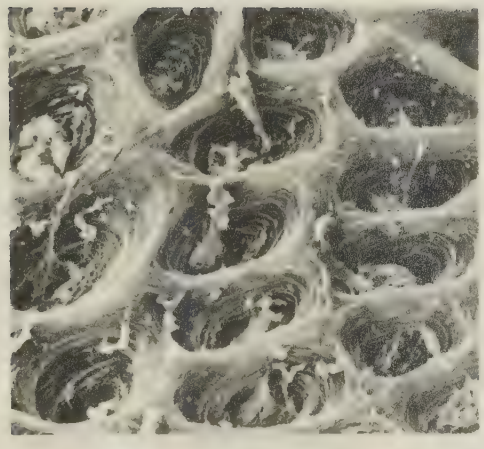
B

Fig. 9. Demonstration of the improvement of the surface preparation technique.

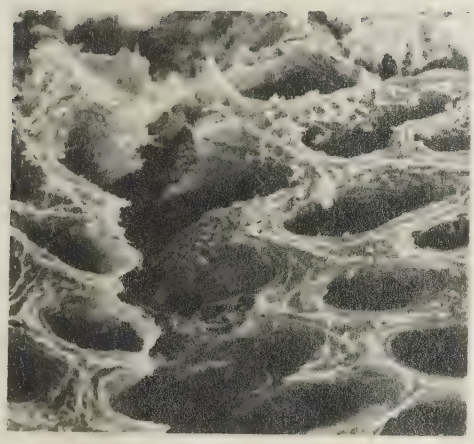
A. Lamina propria of dog scraped with scalpel alone. Note cryptal debris.

B. Lamina propria after finger rub only. The central gap is freeze artifact.

C. Lamina propria after finger rub and scalpel abrasion.



A



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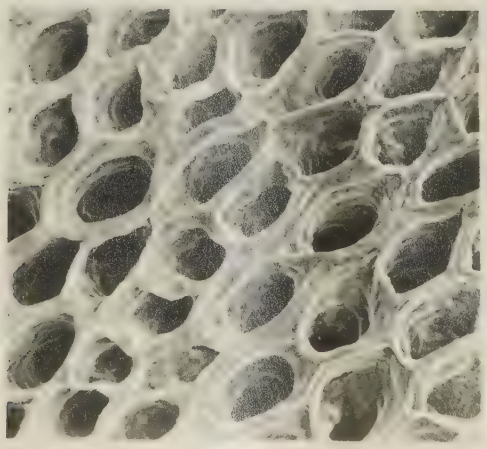


C

3. The specimen was immersed for 10-15 minutes in 2% formalin.

4. The mucosal side of the specimen is rubbed vigorously with the finger.

5. The tunica muscularis is dissected off, separated from the submucosa and discarded.

6. The cleansing of the lamina propria is finished by use of scalpel #11 under direct vision (SLM). To expose the submucosa, the surface is scraped vigorously with a sharp scalpel until such time that the shiny surface of submucosa is observed.

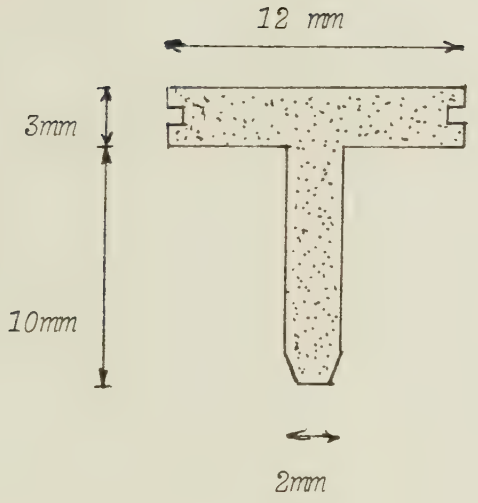
7. Once prepared, the specimen is mounted on a stub and is dehydrated and observed.

The use of the SEM stub, made of stainless steel or aluminum, improved the handling of the tissue for dehydration and SEM preparation. A typical technical drawing of a metal stub is seen in Figure 10. The simple aluminum stubs, as demonstrated in Figure 10a, were mainly used in the latter part of the project for the freeze-drying process. The stub in Figure 10b was mainly used during chemical dehydration in order to prevent dislodgement of the specimen from the stub in the moving fluid.

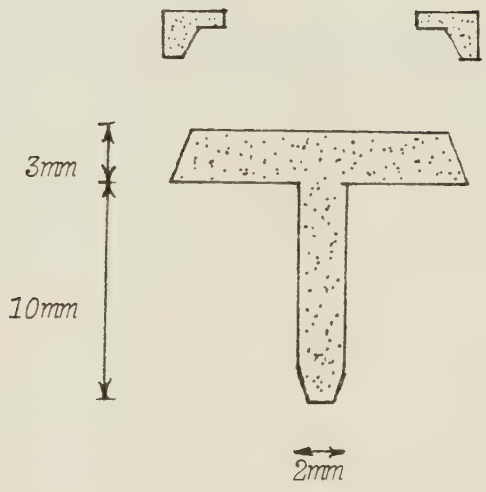
Fig. 10. Detail of the metal tissue stub. A stub made out of steel or aluminum.

a) A technical drawing of a simple stub.

b) Technical drawing of a stub with a collar.



A



B

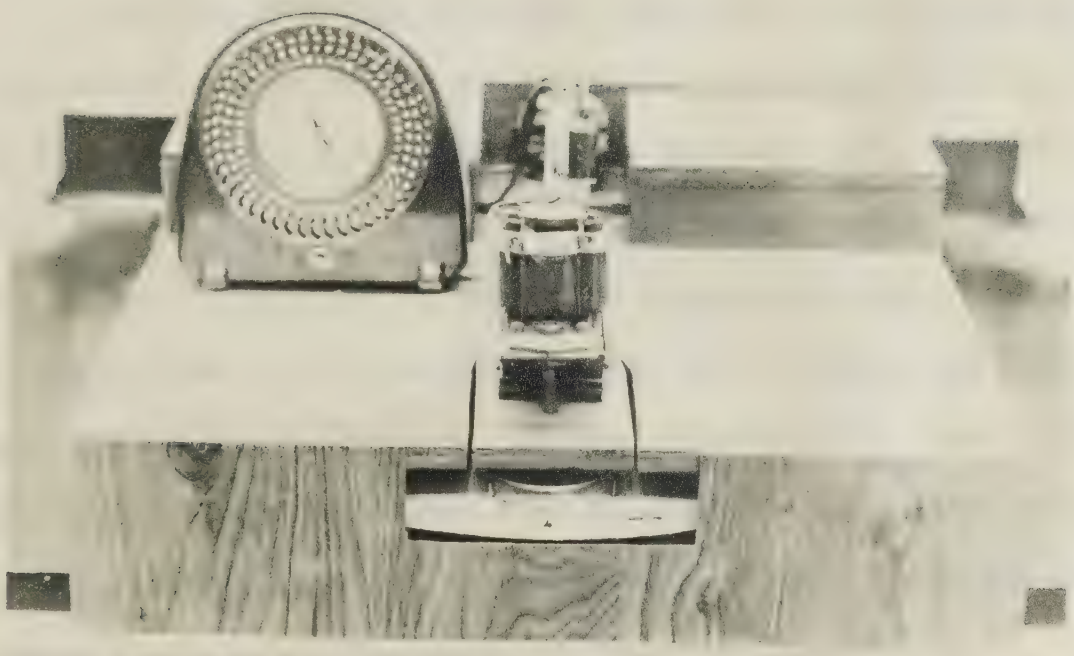
C H A P T E R IV

DEVELOPMENT OF FIXATION AND DEHYDRATION TECHNIQUE

DEVELOPMENT OF FIXATION AND DEHYDRATION TECHNIQUE

The lengthy chemical dehydration process used by Lord et al[50] was subjected to proposed changes as outlined previously. At that time, we were not aware of any alternate method that could be used successfully to dehydrate the lamina propria or submucosa without shrinkage. An automatic tissue processor was designed and built. This simple, functional home-built device was constructed at a fraction of the cost of the commercially available equipment (Figure 11). Utilization of the device markedly reduced specimen contamination as compared to the original technique. The processor made the open-air use of harmful chemicals unnecessary. The time saved by the investigator was the main benefit. The general quality of the specimens improved observably. The device could handle up to 8 to 10 specimens per 24 hours. Soon the rate, at which samples were made available from the hospital and the surrounding laboratories, exceeded the capacity of the processor. The simplicity of the freeze-drying process was very appealing. It was fast. It required only a few tissue-handling steps and it proved to be much more adaptable than the chemical method. With the freeze-drying method, we could process up to 30 samples per day. Having obtained the necessary parts, a prototype of such a device was made. The basic layout of the system is diagrammatically illustrated in Figure 12. This fairly simple device had operated on the principle of keeping the ethane bath at about -40°C in a double coated vacuum vessel. The aluminum stage was pre-cooled to -80°C. The samples mounted on stubs after treatment, at room temperature, were dropped into isopentane pre-cooled to -80°C. The frozen specimens on the stubs were then recovered

Fig. 11. A simple, fully automatic, home-built tissue processor utilized to chemically dehydrate 8-10 samples in 24 hours.



and placed on the pre-cooled aluminum stage. The seal and funnel were put in place and the vacuum was turned on. The vacuum (1×10^{-2}) and the low temperature (-40°C) were maintained for 6-10 hours. The stage had the capability of holding ten samples. The limiting factor was the lack of desiccant in the vacuum vessel. This made the drying time much longer than theoretically possible.

Not only did this primitive setup work, but it produced a spectacular change in the quality of the dehydrated specimens. Figure 13 illustrates the difference between the chemically dehydrated and freeze dried specimens. The use of the freeze-drying principle modified the entire dehydration routine. The fixation of the tissue was limited to ten minutes in 2% formaldehyde, and use of osmium and other chemicals was completely discontinued. Osmium, theoretically, should coat the specimen in order to prevent the charging of the specimen when exposed to the electron beam but, if the specimen is dry enough and coated properly with gold, the osmium treatment is superfluous.

As described earlier, the stub mounted specimens dropped into the pre-cooled isopentane cooled very rapidly to -80°C without isopentane bubbling off. This rapid drop in temperature makes the size of the ice crystals negligible. The pre-cooled stage which was loaded with the frozen specimens on the stubs, was maintained in the ethanol bath at -40°C. After 5-10 hours, samples were allowed to warm up to room temperature while under vacuum. Care was taken not to warm up the samples too rapidly, since a residual amount of water could condense from the air on the relatively cooler samples on the stubs. After 20 runs, fatigue of the makeshift materials and equipment caused problems.

Fig. 12. Diagrammatic representation of the initial freeze-drying apparatus.

1. Aluminum cold stage
2. Anesthetic bag vacuum
3. Specimens on the stubs
4. Plastic funnel
5. Ethanol
6. Vacuum container
7. Cold finger (-40° C)
8. To vacuum pump

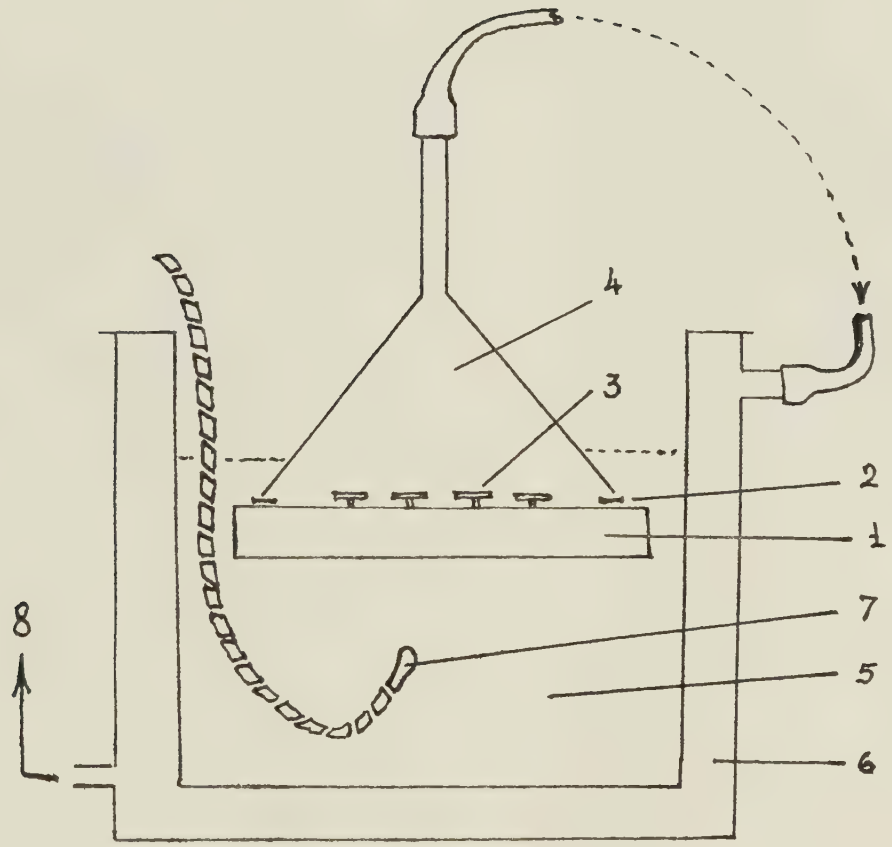

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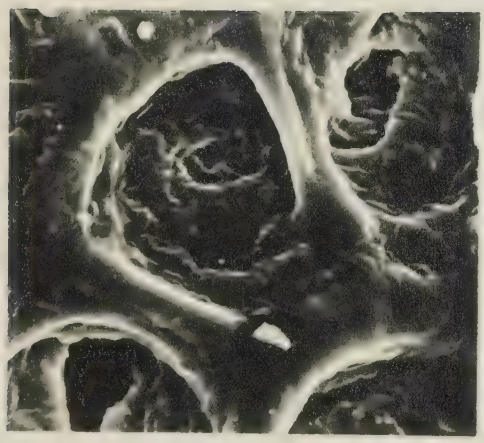
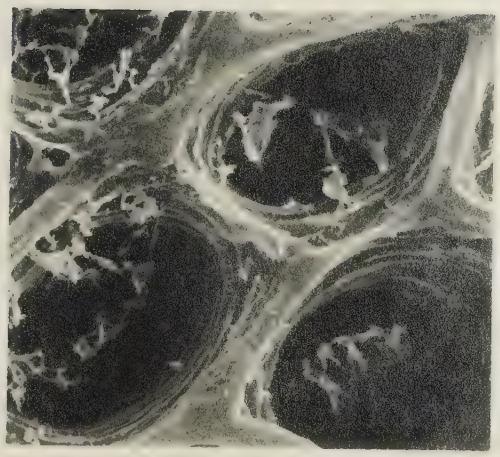
Fig. 13. Comparison between chemically dehydrated and freeze-dried specimens of dog lamina propria.

A. Chemically dehydrated specimen

B. Freeze-dried specimen



A

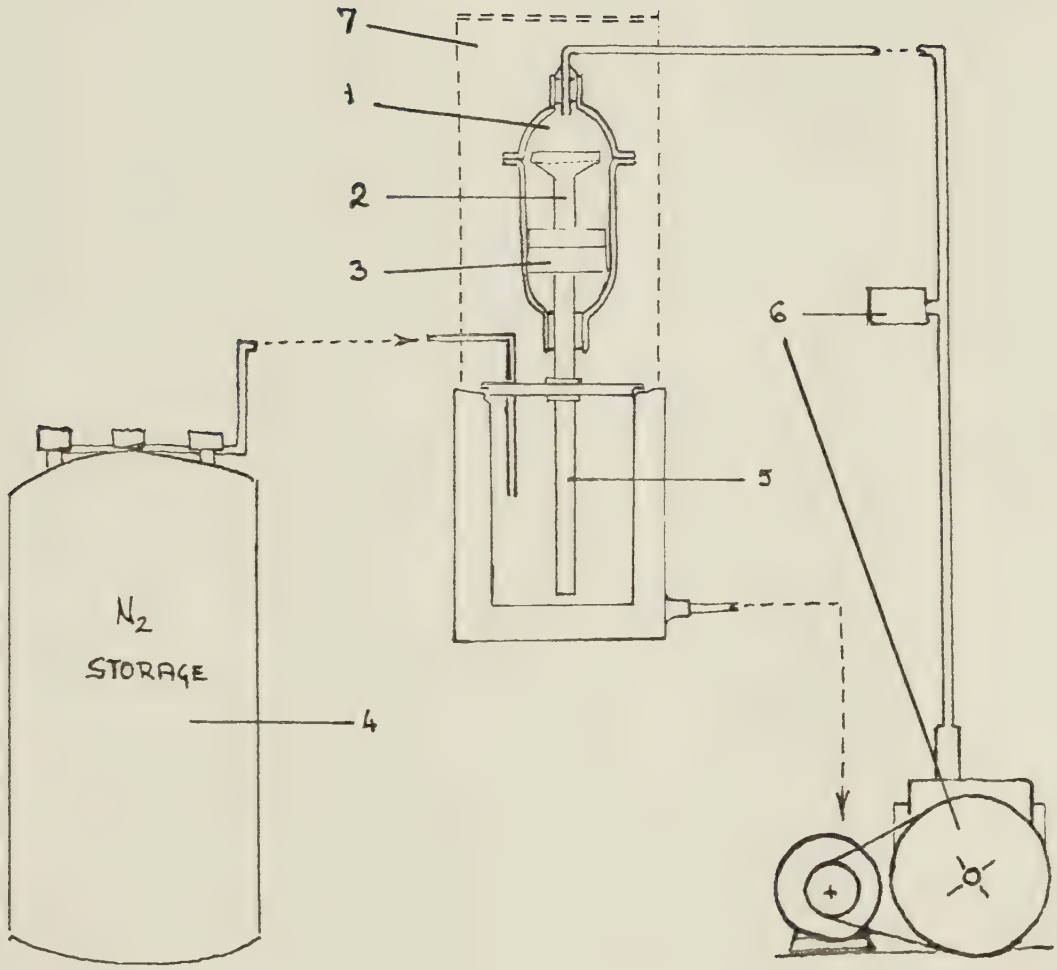


B

A new, more sophisticated freeze-drier was built (Figure 14). The design of the new prototype of freeze-drier is versatile, and can be modified with ease at a later date. It employs the basic principle of sublimation at very low temperature and high vacuum. In its design, it eliminates the troublesome problem of a cold seal. The system incorporates the low pressure-low temperature vessel which is made of pyrex glass. The vessel contains trays for 15-20 g. of desiccant. The vacuum vessel has a glass-to-glass vacuum seal which stays at room temperature. The freeze-drying stage is cooled by a brass rod which is immersed in liquid nitrogen and further cooled by helium to temperatures exceeding -150°C, if necessary. To control and monitor the temperature of the stage, a small thermocouple was installed at the stage level. Temperature control was made possible by installation of a small heater at the base of the glass container. The vacuum of the chamber was monitored by the vacuum gauge system. An increase in the vacuum after two or three hours indicates a relative lack of water in the freeze-dried samples. Where very low temperatures were required, a shield of aluminum foil was fitted over the vacuum vessel so as to prevent the radiation of room heat to the samples. Details of the pressure vessel is documented on the photograph in Figure 15. The use of the described system and use of the present surface preparation techniques produced the excellent images of lamina propria and submucosal surfaces. To illustrate the improvement achieved by development of the described methods, Figure 16 is provided. The questions that arise regarding distortion and artifact introduction, using this technique, is dealt with later.

Fig. 14. Diagrammatic representation of the second freeze-drying apparatus.

1. Pyrex vacuum vessel
2. Brass cold stage with specimens
3. Desiccator trays
4. Liquid nitrogen container
5. Brass cold finger
6. Vacuum pumps and vacuum indicator
7. Aluminum foil shield


7
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STORAGE
4

Fig. 15. Photographic image of the pyrex vacuum vessel.

A. Cold stage with specimens mounted on stubs

B. Desiccant trays

C. Heat conducting cold finger

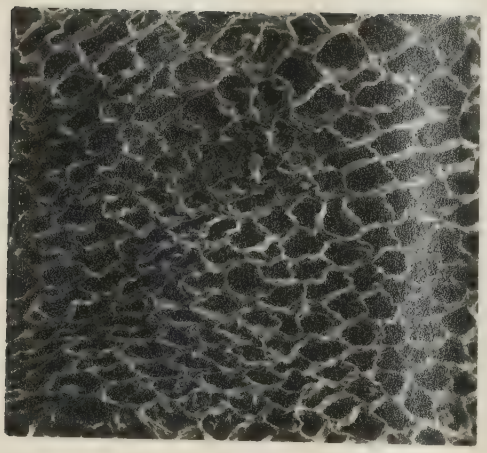
A
B
C

Fig. 16. Three examples of the quality and resolution possible with presented techniques.

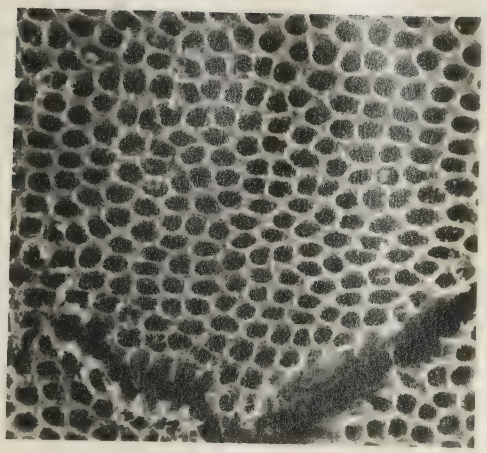
A. Lamina propria in dog distal colon

B. Lamina propria in dog proximal colon

C. Submucosa of rat colon



A



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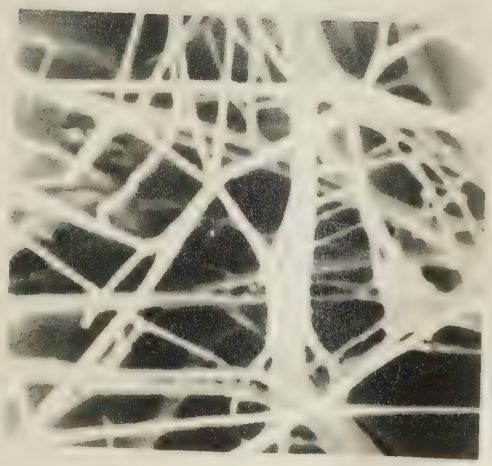


C

C H A P T E R V

OBSERVATION OF LAMINA PROPRIA OF RAT, DOG, MAN

OBSERVATIONS OF LAMINA PROPRIA OF RAT, DOG, MAN

A. General Comments and Observations

The lamina propria in both experimental animals and man has a fishnet-like pattern. This pattern is highly regular and reproducible in the given portion of the intestinal tract. It consists of a repeating series of crypts in the lamina propria. Lamina propria is composed of a rather disorganized network of fibres of variable size. This network is enveloped from the luminal side by a seemingly tough layer of connective tissue, the basement membrane. On the surface, the basement membrane anchors the multitude of cellular elements composing the epithelial layer of the mucosa. This layer is composed of one row of columnar tissue. In order to characterize the lamina propria in an objective manner, the dimensions of the crypts and intercryptic walls were measured. The length of the crypt, the width of the crypt, the length of the cryptal sides, the depth of the crypt and the thickness of the intercryptal walls were measured. The diagrammatic illustration is found in Figure 17. According to this model, the characteristic measurements of the lamina propria of rat, dog and man, are recorded along with the photographic representation of the surface images.

B. Rat Lamina Propria

Six representative samples of lamina propria of rat mid colon have been selected for presentation in this work. The presented examples have been selected from about forty acceptable tissue preparations. Figure 18 depicts six representative images of rat lamina propria as photographed by SEM. One observes that the shape of the crypt may vary. Indeed, the cryptal shape, as observed from the

Fig. 17. Diagrammatic explanation of the measurements of the lamina propria crypts.

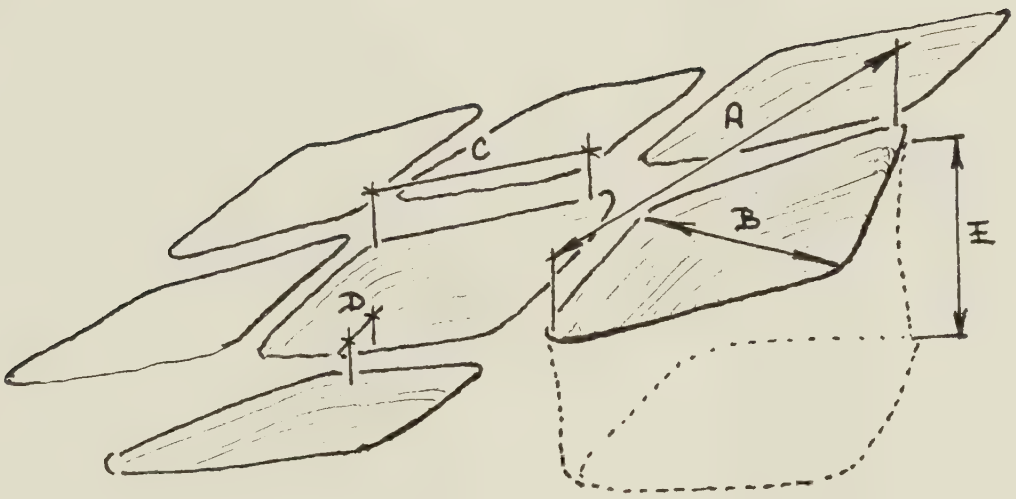
A. Longitudinal measurement of the crypt

B. Lateral measurement of the crypt

C. Length of the sides of the crypt

D. Thickness of the intercryptal wall

E. Estimated depth of the crypt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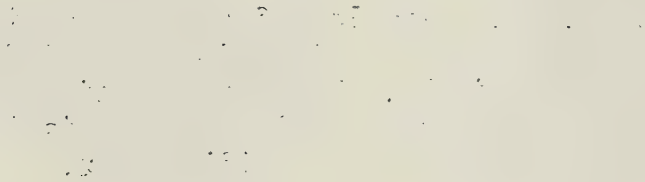
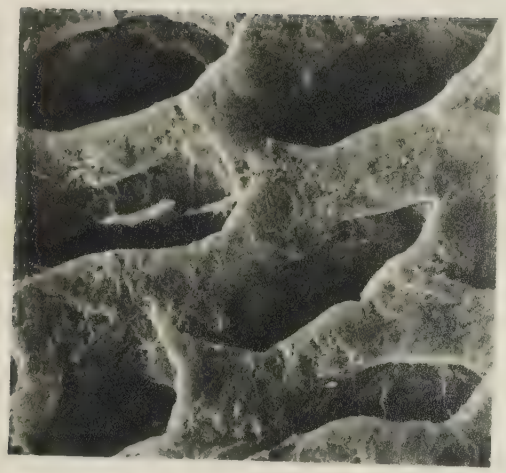


Fig. 18. Six examples of lamina propria of rat obtained from six different animals. The magnification of plates 1, 2, 3, 4, and 5 is 1000x; plate 6 is magnified 400x.



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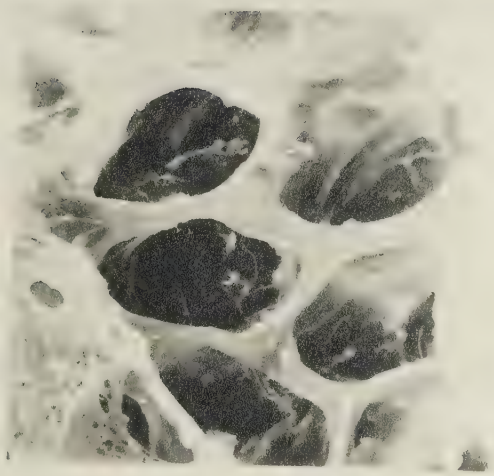

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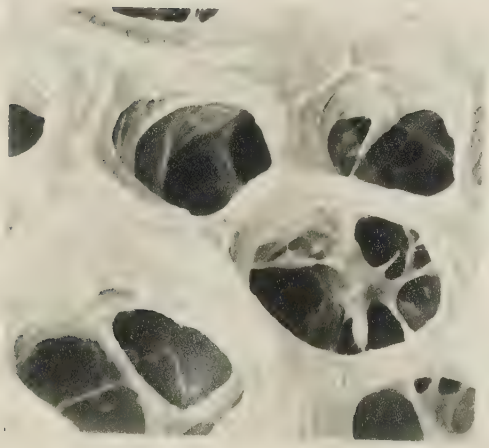

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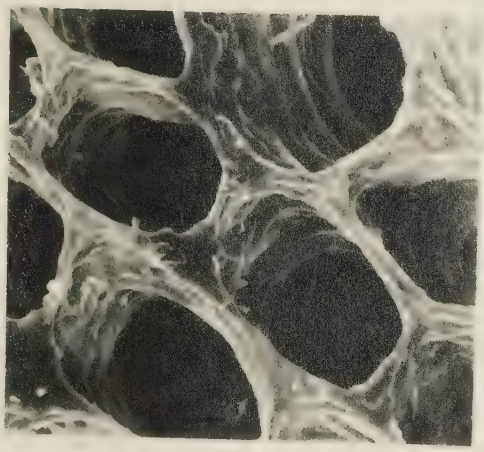

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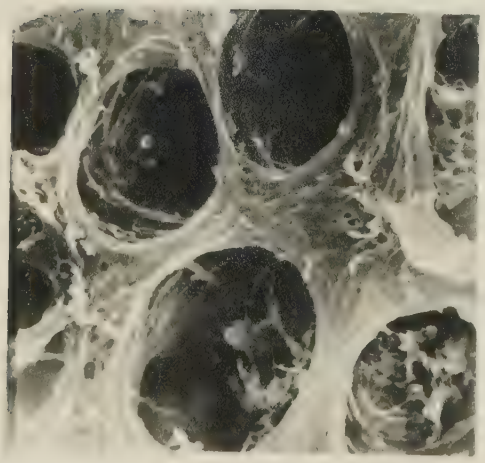

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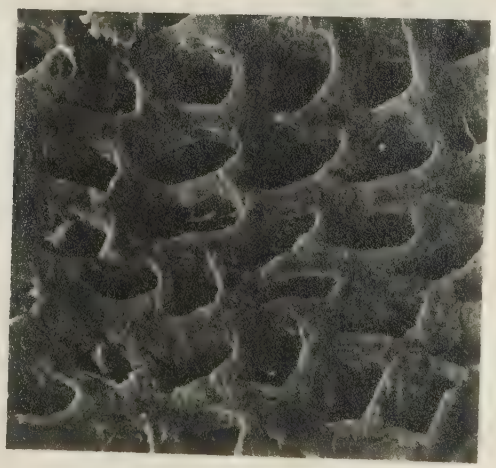


6

surface, varies during preparation of the tissue sample. This change in shape depends on the direction and force of the tension applied to the lamina propria. In plate 1, the tension was applied longitudinally, whereas in plates 3, 4 and 5, it was applied laterally. The other feature that may be noted in plate 3 is the presence of trabeculation, which was not present in the dog or man. The animal donor for plate 3 was healthy. The average dimensions for rat lamina propria were as follows: the length of the crypt was 105 micrometers; the width of the crypt was 55 micrometers; the length of the intercryptal wall was 72 micrometers; the width of the intercryptal wall was 20 micrometers; and the depth of the crypt itself has been estimated by triangulation to be about 60 micrometers.

C. Dog Lamina Propria

Selection of six micrographs of normal lamina propria from the mid colon in the dog has been made from about fifty acceptable, photographed tissue samples. The fishnet pattern is preserved. Only minor differences exist between lamina propria of rat and dog. In the dog, the crypts appear to be larger, the intercryptal walls are thicker and the crypts appear to be of greater depth. There is a greater degree of regularity as compared to the rat lamina propria. Figure 20 represents the six images of six different samples of lamina propria of mongrel dogs. Figure 21 contains the measurements of individual crypts and data for the average measurements of the crypts of dog lamina propria. The intercryptal walls of dog lamina propria appear to be more uniform and smoother than those of rat. The crypts resemble more of an ovoid configuration.

SAMPLE NO.	MEASUREMENTS (in micrometers)				
	A	B	C	D	E
I	120	50	70	20	60
II	100	45	60	20	70
III	100	70	80	10	-
IV	100	60	80	15	50
V	90	60	70	20	60
VI	120	50	70	30	-
	105.0 ±12.3	55.8 ±9.2	71.6 ±7.53	19.2 ±6.5	60.0 ±3.2

Fig. 19. Individual measurement characteristics and average measurement characteristics of normal lamina propria of the female Wistar rat.

A. Length of the crypt

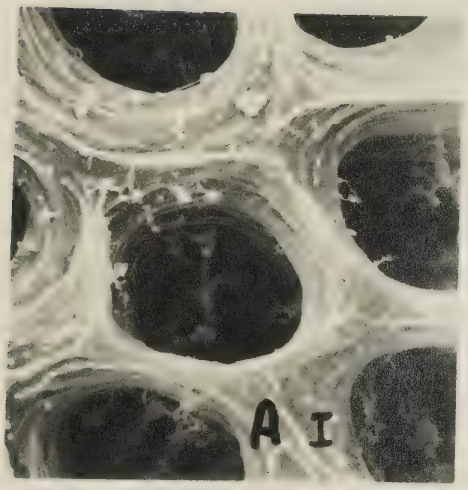
B. Width of the crypt

C. Length of an intercryptal wall

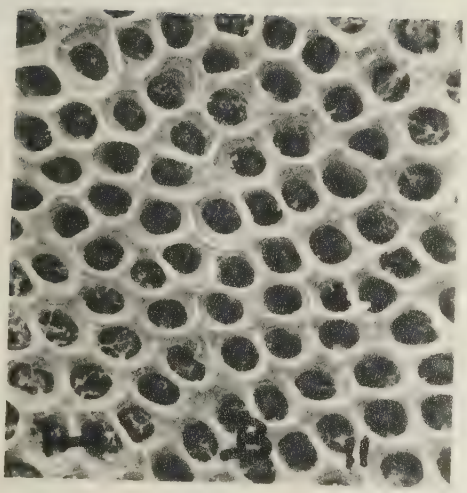
D. Thickness of an intercryptal wall

E. Depth of the crypt

Fig. 20. Six images of six individual tissue samples of lamina propria of six mongrel dogs. Plates 1, 5, and 6 are magnified 1000x; plates 3 and 4 are magnified 400x; and plate 2 is magnified 200x.



1



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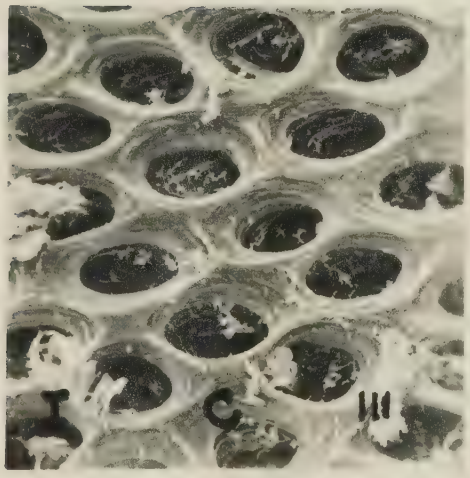

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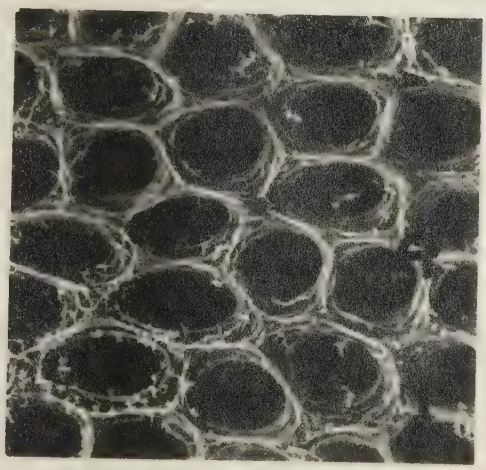

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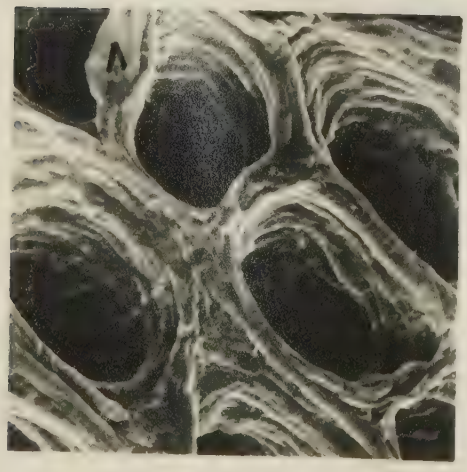

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6

SAMPLE NO.	MEASUREMENTS (in micrometers)				
	A	B	C	D	E
I	100	90	80	40	70
II	100	80	70	30	80
III	130	100	100	40	100
IV	110	90	80	30	-
V	100	70	80	30	80
VI	80	60	60	30	60
	103.3 ±16.3	81.7 ±14.7	78.3 ±13.3	33.3 ±5.2	78.0 ±14.8

Fig. 21. Individual and average information on the normal dog lamina propria.

A. Length of the crypt

B. Width of the crypt

C. Length of an intercryptal wall

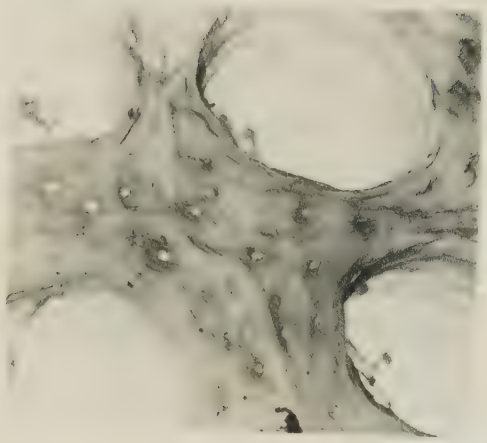
D. Thickness of intercryptal wall

E. Depth of the crypt

D. Lamina Propria of Man

Twenty-one samples of lamina propria were obtained from humans. Of these, about 14 were of acceptable quality. Six were selected for the purposes of this study. Looking at the six examples of the preparation of normal human lamina propria, one has the impression of either smaller crypts or more massive intercryptal walls. Furthermore, the surface of the lamina propria appears to be flat and more even than that in the two experimental animal models. In addition, the regular crypts are dispersed across the lamina propria at regular intervals. The crypts observed in humans, have a perpendicular axis to the surface of the bowel, whereas in the animals, this is not always the case. In Figure 22, which depicts the six individual samples of lamina propria of humans, one will readily notice tiny pores in the lamina propria in most of the samples. These pores are present in the animal colon as well, but they are not as conspicuous. More detailed investigation of these pores will be presented in the latter part of the paper. Figure 23 contains individual and average measurements of the five criteria of size and shape of lamina propria used to characterize and compare.

Fig. 22. SEM images of six different surfaces of normal, human lamina propria. Plate 1 is magnified 1000x; plates 2,3,4 and 6 at 500x; plate 5 at 100x.



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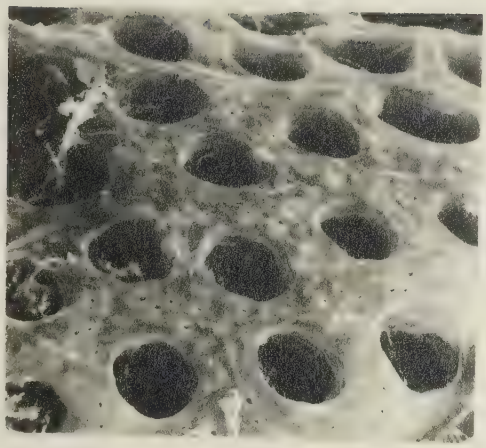

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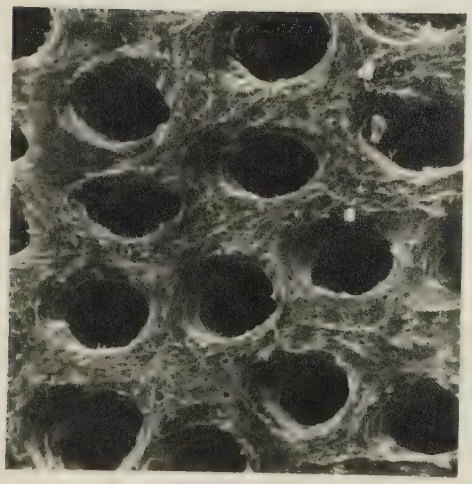

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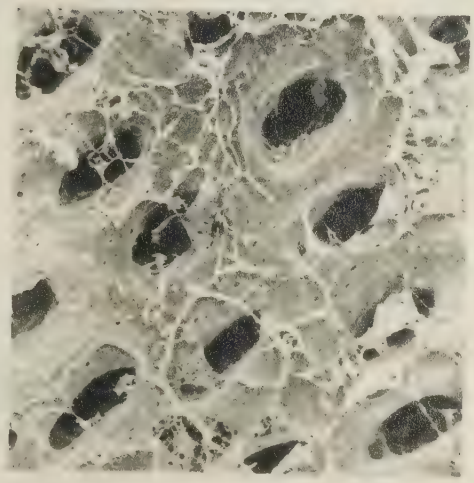

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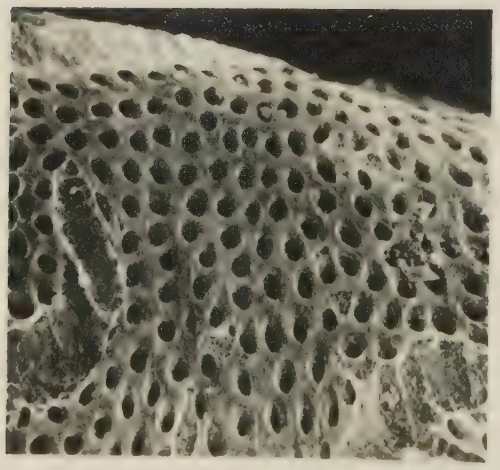

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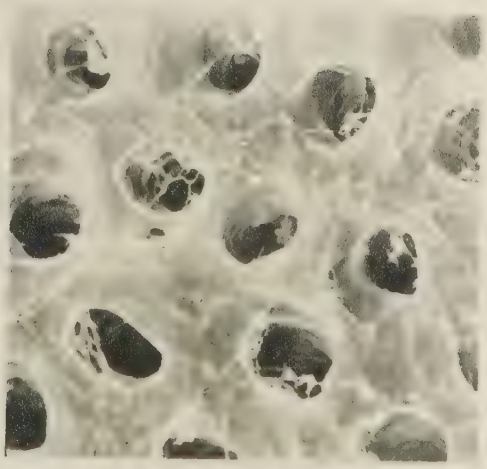


6

SPECIMEN NO.	MEASUREMENTS (in micrometers)				
	A	B	C	D	E
I	120	90	80	40	90
II	100	80	60	50	100
III	110	85	75	50	100
IV	125	75	75	60	-
V	120	70	70	40	-
VI	100	80	80	60	100
	112.5 ±10.8	81.7 ±8.2	73.3 ±7.5	50 ±8.9	97.5 ±5.0

Fig. 23. Individual and average measurements of the dimensional characteristics of normal human lamina propria.

A. Length of the crypt

B. Width of the crypt

C. Length of an intercryptal wall

D. Thickness of an intercryptal wall

E. Depth of the crypt

E. Confirmation of the Identity of Observed Surfaces of Lamina Propria

All specimens observed under SEM were processed through histology. The specimen was embedded in the usual manner after rehydration, then it was cut and stained with H & E for microscopic examination. The SEM observed surface was marked by a dark layer of gold deposited on the free surfaces, and it was identified and correlated to known histological structures (Fig. 24).

F. Characterization of the Normal Lamina Propria in the Three Experimental Models

As indicated from the examples presented, the lamina propria of the three models is very similar in most aspects. There are only minor dimensional differences. The differences between the three models are: 1) the more random arrangement of the crypts in the rat lamina propria; 2) the very thin intercryptal walls of the rat lamina propria; 2) somewhat irregular dimensional characteristics of rat lamina propria; 4) the dog lamina propria is an interface between rat and human lamina propria, and 5) the human lamina propria has deep, circumferential equidistantly-spaced crypts divided by thick, uniform intercryptic walls.

G. Substrate of Lamina Propria

The lamina propria is composed of numerous multi-directionally-oriented fibres and fibre bundles. These are enveloped from the luminal side by the lamina propria. Figure 25 illustrates visually the nature of the bundles composing the mass of lamina propria. On the muscularis mucosa side, the bundles interlace with the smooth muscle forming a tight bond.

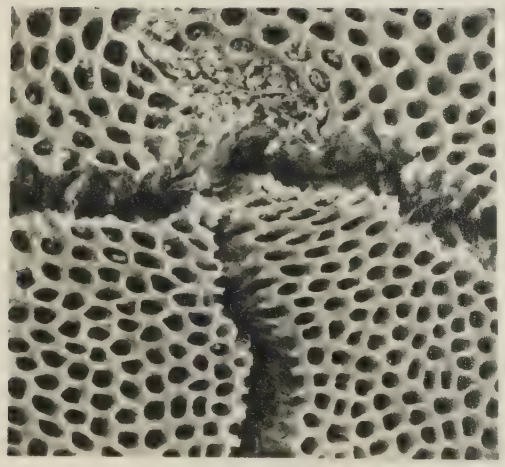
Fig. 24. Identification of the observed surfaces using H & E sections of the viewed specimens.

A. SEM micrographs of lamina propria

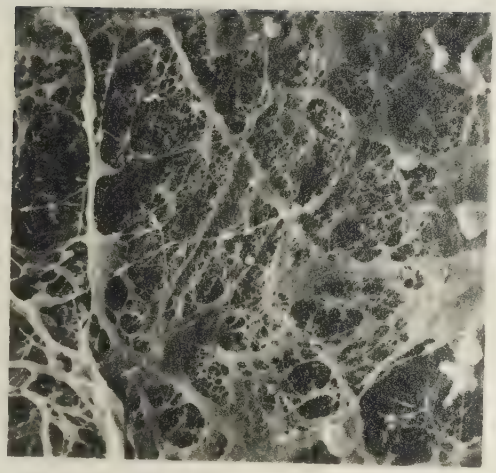
B. Corresponding light microscopy photo

C. SEM view of submucosa

D. H & E section of the same specimen



A



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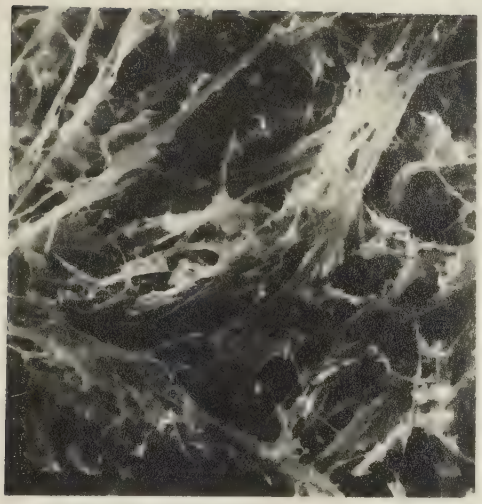
C

D

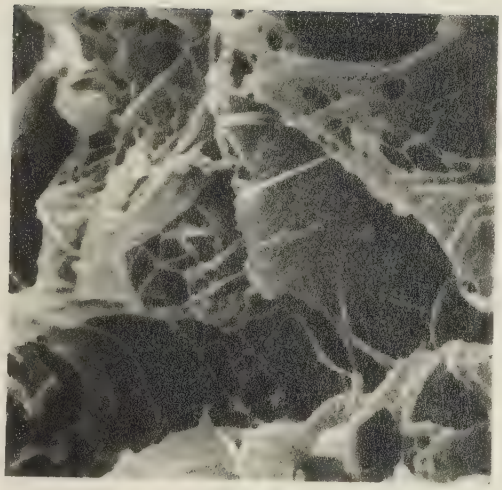
Fig. 25. Three images of the architectural arrangement of collagen fibers within the mass of lamina propria.

A. and B. Random organization of disordered collagen bundles and aggregates.

C. Basement membrane partially stripped.



A



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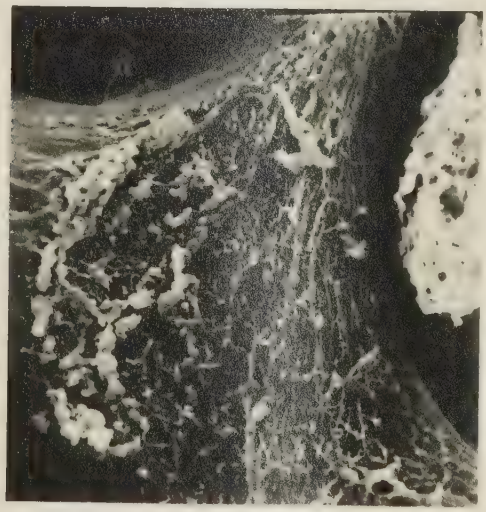


C

CHAPTER VI
NORMAL SUBMUCOSA

NORMAL SUBMUCOSA

The tight adherence of muscularis mucosae to the luminal surface of submucosa made its study difficult. Under SEM the submucosa appears as a layer composed of a multitude of fibers of collagen and collagen bundles. These fibers and bundles are multidirectional and appear to be arranged in loosely delineated fiber layers. During the investigation three types of fibers have been noted. Class I fibers are the thin, long fibers with average dimensions of 2,500 Å - 7,500 Å in diameter and 60 - 100 μm in length. Class II are the intermediate fibers, and are short, stubby fibers with average dimensions of in diameter and 5 - 15 μm in length. Class III, are long, thick fibers with interconnecting fibrils emanating from them. The dimensions of these fibers are quite variable but may be found in the range of 10,000 Å to 15,000 Å in diameter with the length exceeding 100 μm.

A. Rat Submucosa

The submucosa of rat colon has the general features described previously. Figure 26 illustrates six samples of submucosa fibers taken from six different animals, and shows the large variability of appearance as seen by SEM. Most of the ground substance on the surface has been removed and the fibrillar structure of the collagen fibers and bundles is clearly seen. In micrographs 1 and 2 interconnections of the fibers are quite evident. Plate 3 demonstrates the presence of the Class III fibers along with Class I and II. Class III is the thick fiber running diagonally through the center of the image in plate 3. It is interlaced and connected to Class I fibers, below the big fiber, and the Class II fibers are in the top left-hand corner of plate 3.

Fig. 26. Normal submucosa of rat.
Magnification of plates:

1 2000x

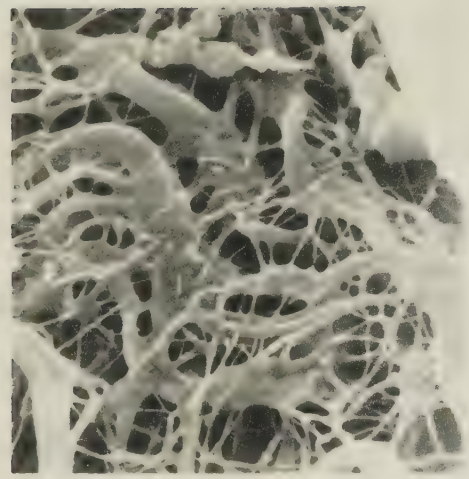
2 1000x

3 500x

4 200x

5 200x

6 100x



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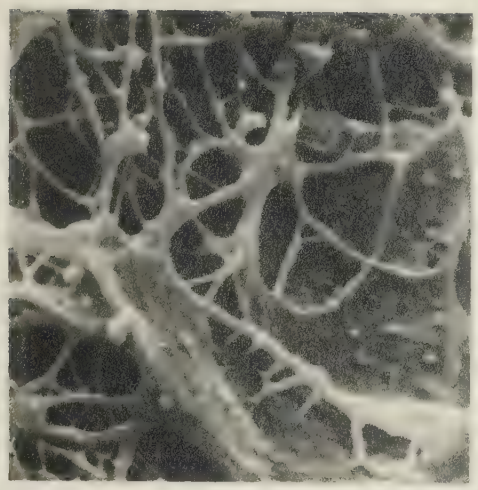

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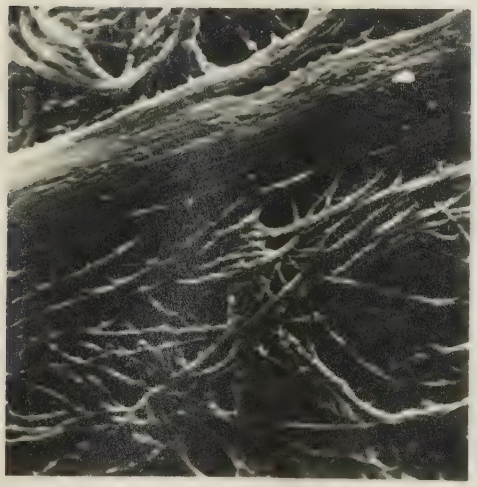

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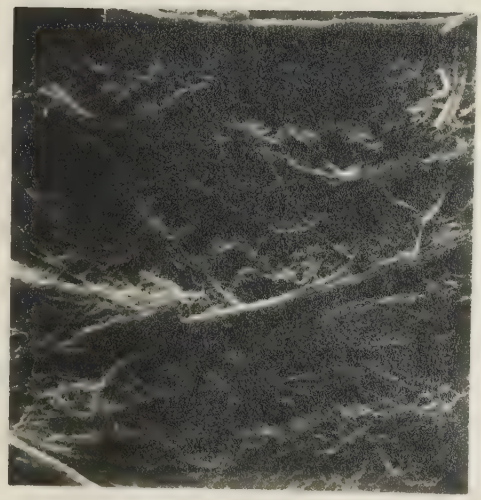

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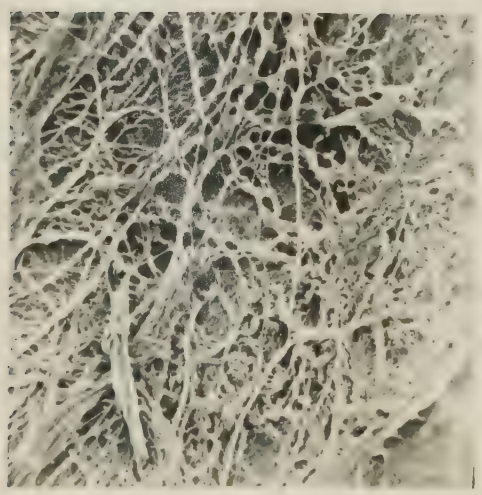

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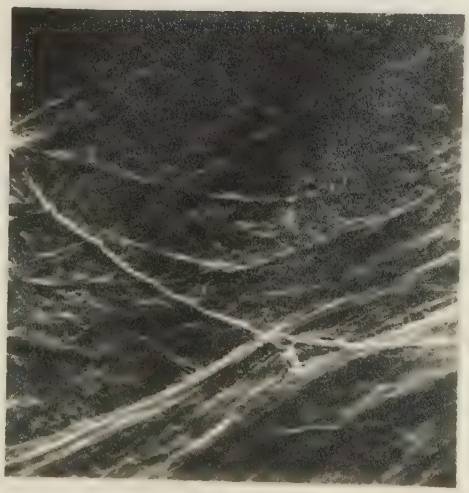

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Similarly, in the remaining plates, different types of fibers and fiber bundles can be recognized.

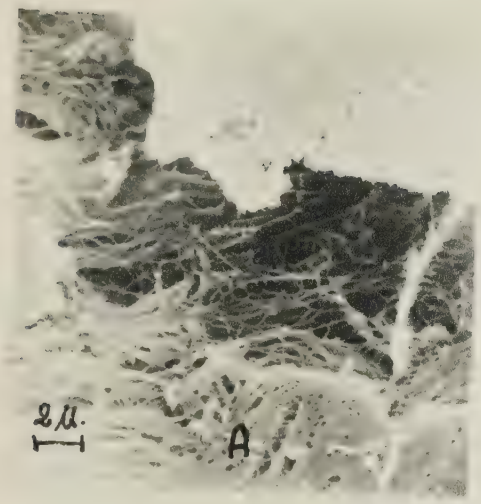
B. Dog Submucosa

The submucosa obtained from the thirty tissue samples of canine colonic tissue have been investigated and photographed. Six individual samples have been selected to demonstrate the variability of the submucosal image. In Figure 27, plate 4, one can readily recognize the conglomeration of fiber bundles running in one direction in one layer, and in the other direction in the layer below. Most of these fibers are Class I and II.

C. Submucosa of Man

Figure 28 contains six examples of human colonic submucosa. As mentioned, human colonic submucosa has no distinctive features. Figure 28 demonstrates the concept of a multilayered submucosa. In plate 1, bundles of fibers run almost perpendicularly to each other at the different layers. The same is the case for plate 2, plate 4 and where the bundles of the three classes crisscross in different directions. However, the bundles of one layer seem to run unidirectionally. In Figure 28, plate 4, at magnification of 2000x, the large bundles appear to interconnect with the bundles running in the layer above and below. The interconnecting fibers of the submucosa will be examined later. From the point of view of measurement characteristics, the human submucosa is identical to rat and dog submucosa structurally and in dimensional detail.

Fig. 27. The normal submucosa of dog.
Plates 1-6 magnified 500x



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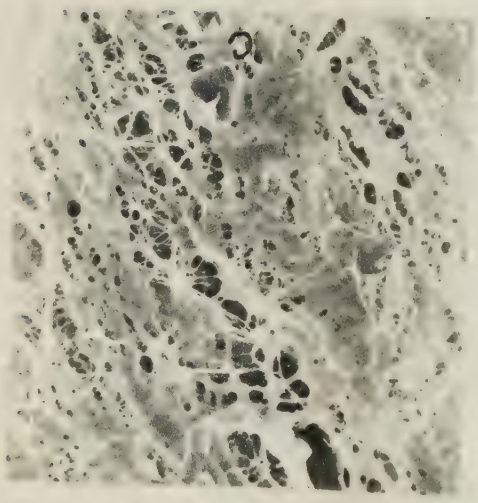

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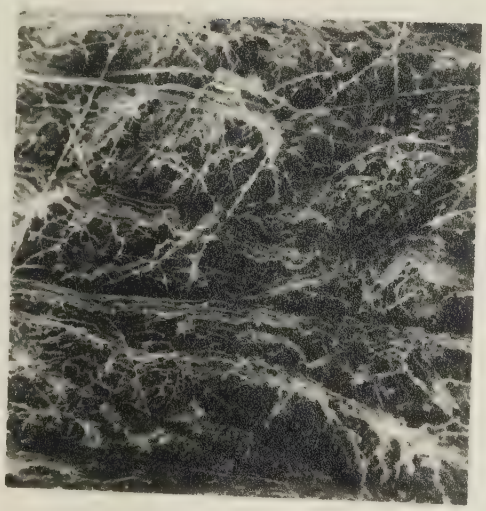

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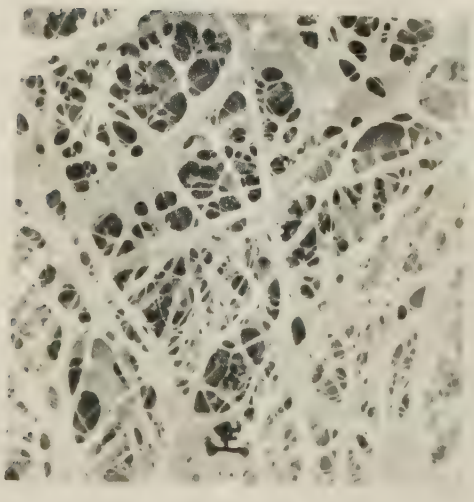

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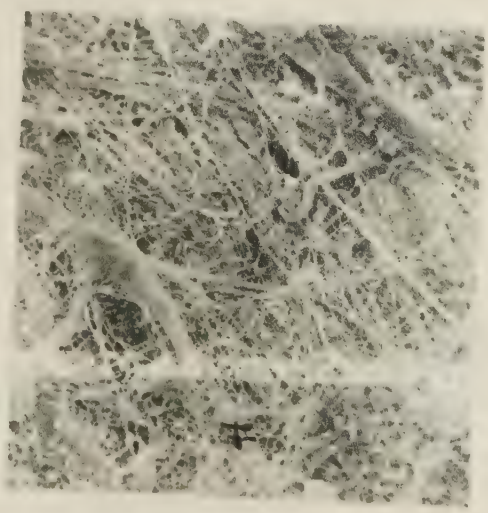

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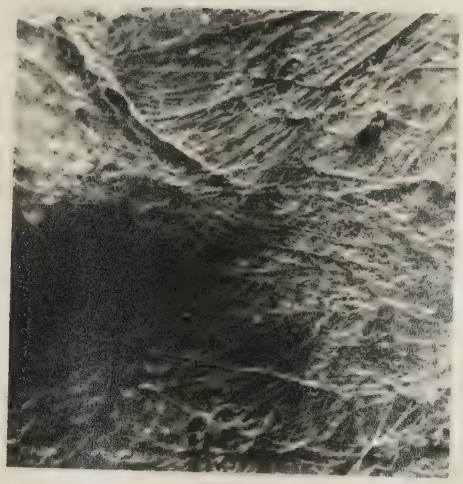
Fig. 28. The normal submucosa of man. Plates 1, 3, and 5 are magnified 400x; plates 4 and 6 are magnified 2000x; and plate 2 is magnified at 100x.



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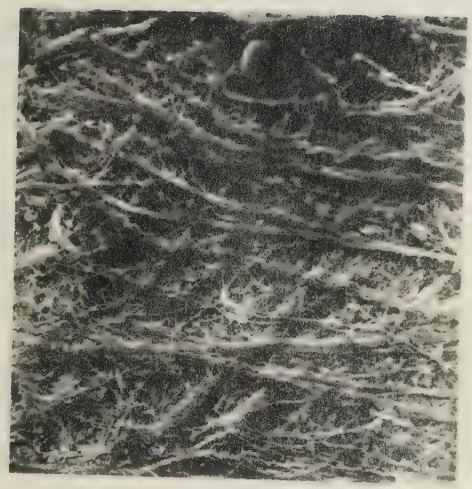

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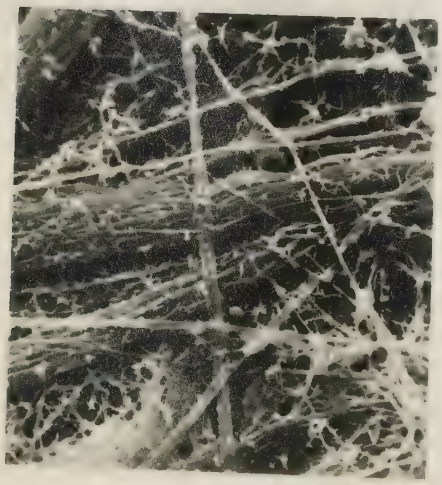

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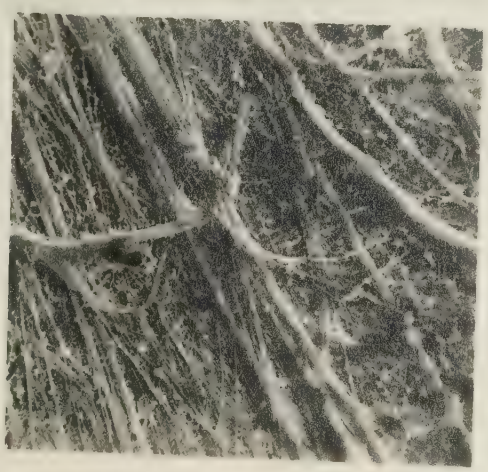

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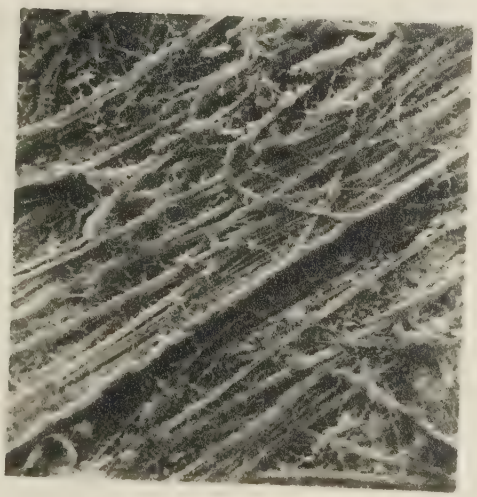

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D. The Crosslinking of Submucosal Fibers

After careful preparation and gold plating, the fibers of human colonic submucosa were photographed at high magnifications. Figure 29 contains six examples of Class I fibers interconnecting with each other by individual collagen fibers. The lobulated fibers may represent damaged ends of collagen fibers which have been either disrupted or chemically denatured during preparation of the sample. These are present on most specimens examined. The important feature in these images, obtained by high magnification SEM, is that the Class I fibers are interconnected with each other by means of single, short collagen fibers. This may explain the limited slip, allowing some elasticity in a tissue composed of a rigid fiber such as collagen.

Fig. 29. Visual demonstration of crosslinking of submucosal collagen fibers.

Magnification of plates:

1 15,000x

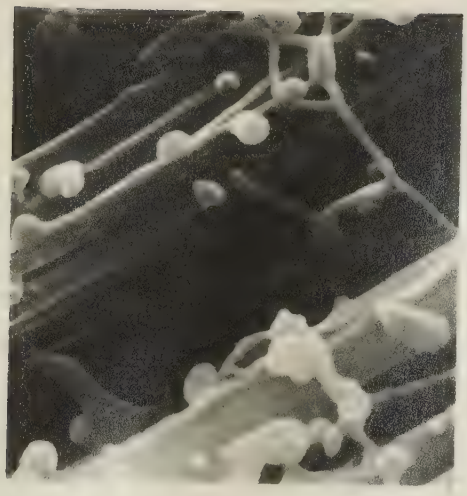
2 15,000x

3 20,000x

4 30,000x

5 20,000x

6 20,000x



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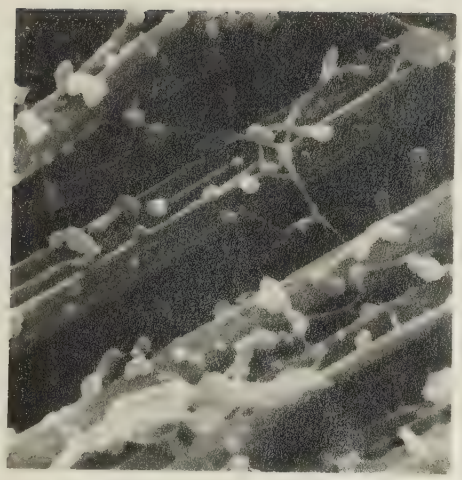

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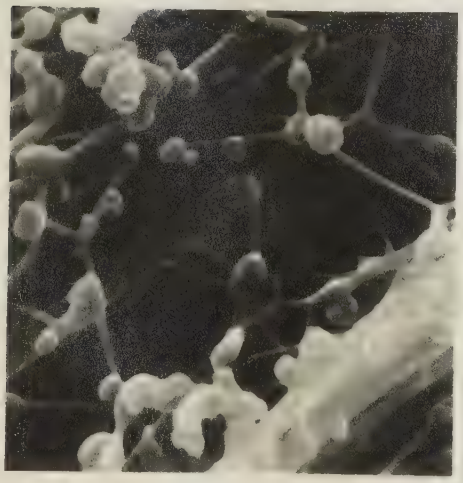

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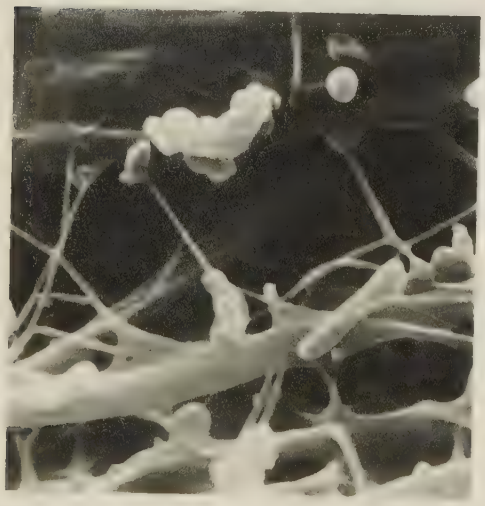

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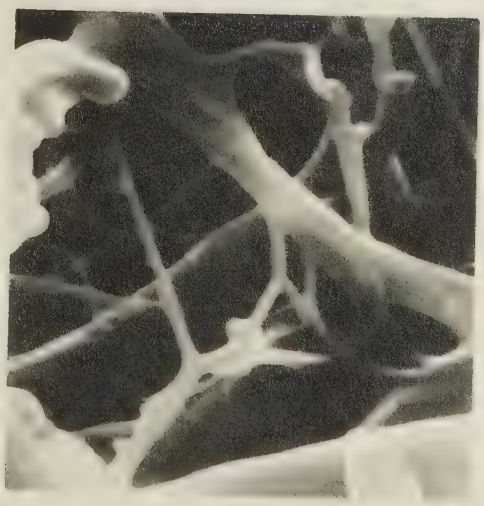

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C H A P T E R VII

DISCUSSION OF LAMINA PROPRIA, SUBMUCOSA AND THEIR STRUCTURE

DISCUSSION OF LAMINA PROPRIA, SUBMUCOSA AND THEIR STRUCTURE

A. General Description of the Lamina Propria

In the three species studied, the surface geometry of the lamina propria may be, in part, a result of the orderly arrangement of the Crypts of Lieberkühn as well as the result of the basement membrane texture covering the lamina propria substrate. However, this argument may be true in reverse as well. The pattern of the mucosal surface arrangement follows the underlying shape of the lamina propria. The longitudinal and lateral dimensions, measured in micrometers, vary with the direction and the magnitude of the tension on the network of the lamina propria.

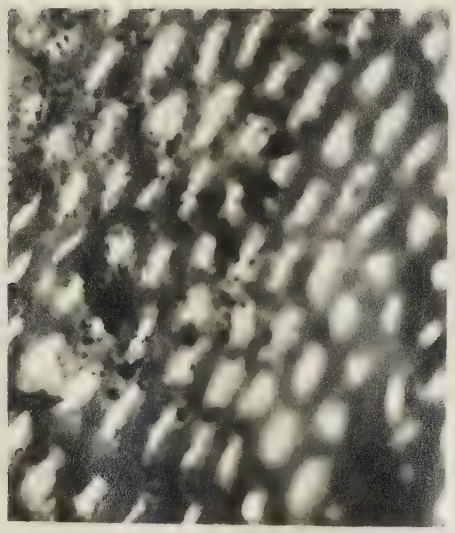
B. Optimal Packing Arrangement

One may ask: Why did the hexagonal or diamond-shaped arrangement develop in the lamina propria of rat, dog and man? The hexagonal arrangement seems to be Nature's more efficient manner of packing the most volume into a confined surface or space. This may be a simple explanation, but it is supported by the frequent occurrence of this shape in many biological forms. Also, the hexagonal arrangement explains the geometry of distention of tissues such as lamina propria. Since lamina propria is made of collagen, which is non-distensible, the geometry of parallelograms explains the distensibility in a longitudinal direction while contracting in the lateral direction. This fact may be verified very simply by manipulating the lamina propria on the stage of the scanning light microscope while cleaning the lamina propria with a knife blade (Figure 30). The mass of the lamina propria itself is composed of a relatively loose reticular network of collagen

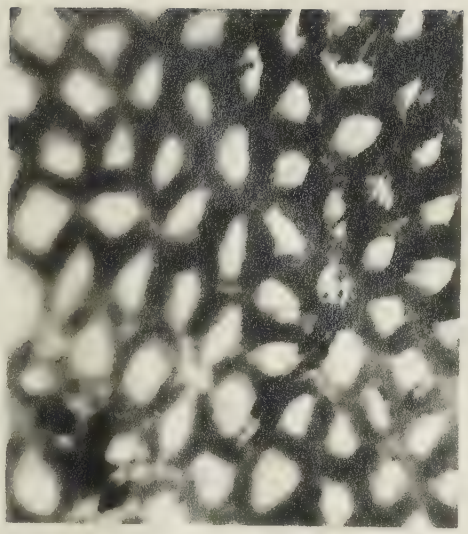
Fig. 30. Demonstration of the parallelogram arrangement in the architecture of the lamina propria.

A and C - lamina propria stressed longitudinally.

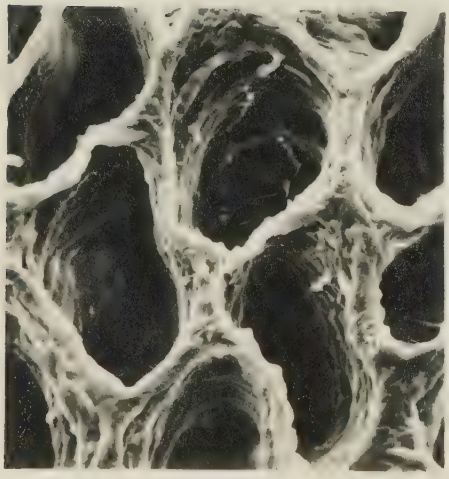
B and D - lamina propria stressed laterally.



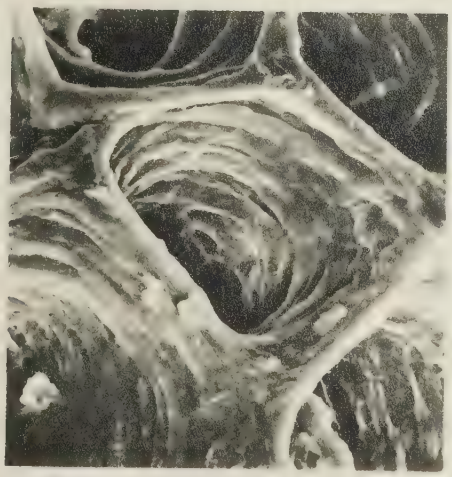
A



B



C



D

fibers. These fibers are short (40 μm), and they branch profusely and interlink with each other.

Figure 31 includes several examples of the above-mentioned random arrangement of collagen. During the observations of the substrate of lamina propria, expecially in the vicinity of the basement membrane, one would expect to find a reasonable amount of cellular elements. The presence of fibroblasts, microphages and eosinophils has been reported by Trier. During our studies, no cellular components were noted in the exposed lamina propria. This may be due to the fact that the cells are embedded in the matrix of lamina propria, and out of the view of the scanning electron microscope, but also, the possibility exists that the observer was unfamiliar with the surface appearance of the cellular membranes, or that the cellular elements were removed during processing.

C. Interpretation of the Dynamic Structure of Lamina Propria and Submucosa of the Gastrointestinal Tract

Both the lamina propria and submucosa are composed of collagen bundle embedded in ground substance. Due to its chemical structure, collagen does not distend appreciably. However, when collagen is conglomerated into sheets of tissue, such as lamina propria or submucosa, these layers of tissue are distendible to a remarkable extent. These layers of lamina propria and submucosa can accommodate drastic changes in circumference without destruction of the structural order of the collagen layer.

As mentioned, collagen existing in the lamina propria is of a reticular nature. By virtue of the geometric, fishnet configuration

(parallelograms), the non-distensible connective tissue can accommodate changes in the diameter and length (Figure 30).

A row of columnar epithelium lines the mucosal surface of basement membrane covering the lamina propria. Each Crypt of Lieberkühn has a small space communicating with the volume of the lumen of the bowel. As the peristaltic wave passes through the length of the colon, the diameter of the lumen varies. This change of diameter changes the pattern of the lamina propria skeleton -- the fishnet -- which in turn changes the volume of this space trapped in the Crypt of Lieberkühn. Thus, the peristaltic movement not only moves the contents of the lumen, but also ventilates the Crypts of Lieberkühn.

D. Distensibility of the Submucosa

As illustrated previously, the submucosa is composed of multiple layers of multidirectional collagen fibers and bundles. The majority of the fibers intercommunicate with the neighboring fibers along its length. These crosslinks, as seen in Figure 29, allow for a limited slip of the individual collagen fibers or bundles past the neighboring bundles. The summary effect of a large number of fibers slipping past each other, results in a significant distension or compression of the collagen matrix of the submucosa. Since no fiber has been destroyed or disconnected, its integrity and strength is not adversely affected when distended within physiological limits. During the observation of several micrographs of submucosa of dog, the impression was created that the submucosa is actually composed of multiple lamellar plates put together from a large number of collagen fibers running parallel. The lamellae are, in turn, interconnected by short fibers. This may

explain the ability of submucosal tissue to distend significantly without loss of strength or integrity.

E. Interrelation Between the Lamina Propria, Muscularis Mucosae and Submucosa of Colon

In all three species -- rat, dog, man -- lamina propria, muscularis mucosae and submucosa appear to act as a unit. These three layers are bonded together very closely. It is difficult to separate the lamina propria selectively from the muscularis mucosae, and it is almost impossible to scrape off the thin, muscular layer from the lumenal side of the submucosa, even when using a sharp scalpel. The interposition of the muscularis mucosae provides a degree of independent motility between the lamina propria and submucosal layer.

During preparation of the technique for cleansing the lamina propria and submucosa, it was discovered that the lamina propria was not as flimsy as initially believed. It requires vigorous abrasion with a scalpel to remove the crypts from the muscularis mucosae. The adherence of the muscularis mucosae to the underlying submucosa is best demonstrated when one attempts to remove it. This is a scalpel breaking experience. Possibly the combined strength of this laminae of three layers, as seen physiologically, possesses more strength than the simple addition of the individual layers (Figure 31).

Changes in the muscularis mucosae (edema, tension increase) may affect the blood supply and lymphatic drainage of the lamina propria and epithelial part of the mucosa. Since the vessels and lymphatics must pierce the muscularis mucosae and submucosa, the blood supply will be affected by the change of tension in these two layers. In areas

where the submucosa is thinned out, by either the vascular plexus or lymphatic tissue, the intraluminal pressure, which is translated into surface tension, stresses the fibers of the submucosa in the areas where the submucosa is thinnest and, therefore, a smaller number of fibers share the induced stress. This situation is likely to occur at the site of the submucosal vascular plexuses. By increasing the tension, the perforating vessels reaching these plexuses get "choked off". Thus, ischemic insult is added to mechanical stress.

F. Use of Human Cadavers for Bowel Wall Specimens

Resection of the colonic and gastric specimens is a common place occurrence in operating rooms. To obtain a section of normal esophagus, jejunum, duodenum and ileum from humans is much too rare to use as a source for experimental work.

1) Advantages of cadaver specimens. An obvious advantage of this source of human specimen is the availability and relatively plentiful supply of specimens. On most occasions, donors obtained in this manner had accumulated a significant hospital record, making correlation of pathology or abnormality much easier than for the fresh surgical specimens.

2) Limitations and disadvantages of cadaver specimens. The most significant problem with this method of collecting specimens is post mortem autolysis. In contrast to the surgically removed portion of the intestine, the bowel in the fresh cadaver undergoes a gradual reduction of temperature instead of a rapid drop, as is the case in the surgically removed specimen. Thus, the rate of enzymatic activity of lysosomes in the epithelium and in the other layers of the intestinal

wall is very rapid for the first two to four hours, since the temperature of the core of the fresh cadaver does not drop significantly in the first three or four hours. During the course of the study we have investigated this possibility. We have collected specimens of gastric, duodenal, jejunal, ileal, caecal and colonic specimens from 21 cadavers. Nine cadavers were over 12 hours old, eight cadavers were over 8 hours old and four cadavers were three to four hours old. It was soon found that the specimens removed from the cadavers that were 12 hours or older were useless for all practical purposes. Some of the specimens from the 8-10 hour cadavers were usable, but the quality was questionable. The specimens removed from the few fresh cadavers (less than five hours old) provided the electron scanning images comparable in quality to the post-operative specimens. From this somewhat limited experience, we have concluded that the cadaver specimens can be used for the investigations under scanning electron microscopy, if the specimens were removed within four to five hours after death. This, however, poses some problem practically, due to the fact that most cadavers do not get into the autopsy room within that limited time.

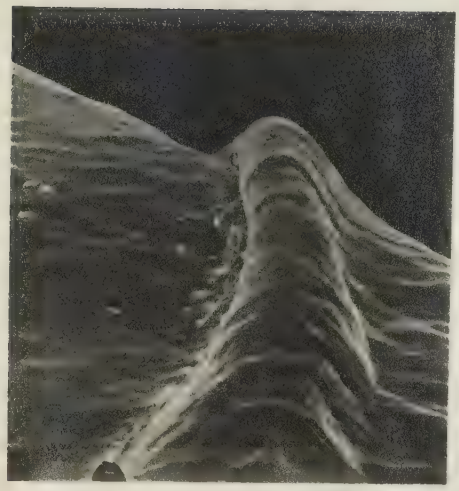
G. The Micropores on the Surface of the Lamina Propria (Stomata)

The coverings of the lamina propria of man (the basement membrane) demonstrated the presence of minute pores. These tiny 1-2 μm oval pores were noted in the relatively tough and impervious basement membrane, especially on the peaks of the intercryptic walls (Figure 32). These pores have an organized construction, they are usually bipartite, and, on close examination, the pores are found on the surface of the basement membrane in both the dog and rat as well as humans. However,

Fig. 31. Muscularis mucosae and submucosa of the dog midcolon.

A. Muscularis mucosae covering collagen fibers of the submucosa.

B. Exposed submucosa (right) is in close contact with muscularis mucosae (left).



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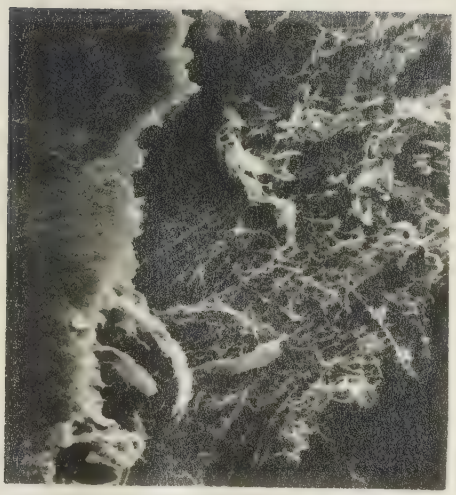


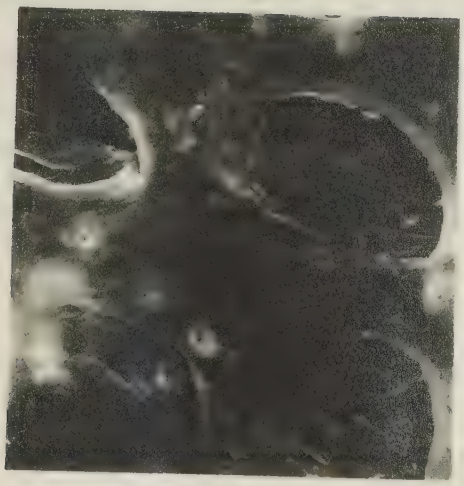
B

Fig. 32. Micropores (stomata) of the lamina propria of human colon.

A. Note small pores on the surface of the lamina propria (mag. 500x).

B. Detail of the pores (mag. 2,000x).

C. Microstructure of one of the openings (mag. 10,000x).



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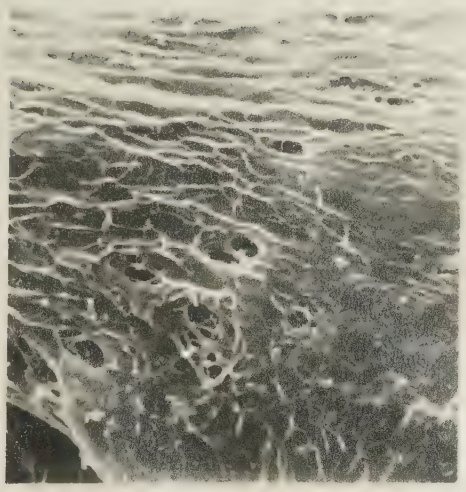

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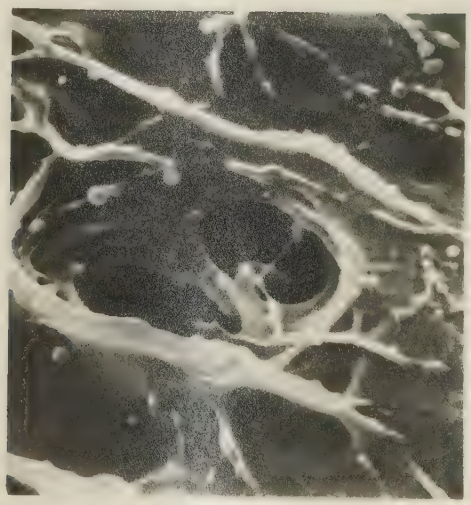

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the frequency and density of these pores are smaller in the experimental animals than in man. The significance and the function of these pores is not known. It was noted that they are located at the base of the epithelial cells by the depressions of the columnar cells in the basement membrane. In the literature, mention was made of occasional discontinuity of the basement membrane of the lower wall. Perhaps the pores (stomata) are corollary to the wall discontinuity of the basement membrane as found by TEM.

C H A P T E R VIII

DISCUSSION OF THE TECHNIQUE

DISCUSSION OF THE TECHNIQUE

A. Discussion and Evaluation of the Surface Preparation Technique

The surface preparation technique used for this study is quite satisfactory (Page 27). It is relatively fast, simple, reliable and it does not damage the colonic specimen significantly. This technique has been used to produce the majority of micrographs presented. However, there are potential problems with the technique.

The technique of crushing the epithelial lining with the finger to achieve a clean exposure of the large areas of the dog or human submucosa, requires brute force to separate the lamina propria and muscularis mucosae. The difficulty seems to lie in the fact that the lamina propria is much sturdier than originally believed. The tight adherence of muscularis mucosae to the submucosa makes the abrasion technique quite difficult. The submucosa has to be separated from the bulky tunica muscularis, and it must be applied to a very smooth surface, and it must be scraped with a straight blade of a very sharp scalpel. Use of light refraction of the surface is quite useful. When the muscularis mucosae is present on the surface of the submucosa, the light reflected has a ground glass appearance. When the submucosa is reached, the light is reflected as from a glistening surface. The technique is quite satisfactory and, with some experience, it can produce excellent results. Modification of this technique will be necessary in the future, since many of the specimens for investigation of clinically significant disease of the colon will be of minute size, probably 1-2 millimeters in diameter, as is the case in endoscopic biopsy. With such small specimens, it would be almost physically

impossible to treat the surface of lamina propria and separate submucosa from the overlying layers. The other problem of this crush and scrape technique has appeared when a large number of gastric, duodenal, ileal and jejunal samples have been treated. In a majority of the cases the villi, which should contain some lamina propria skeleton, have been sheared off along with the collagenous skeleton during the stage of finger rubbing and scraping. This probably occurred because of the tight adherence of the columnar cells to the villus and the relatively small cross-section of collagenous attachment of the skeleton of the villus to the underlying lamina propria. However, when a mild degree of autolysis has occurred, the cells have separated quite readily and many collagen-containing villus skeletons have remained for observation. The ideal technique for this purpose would be to detach the cells by desmosomal disruption and remove the cells by washing, using a minimal amount of mechanical force.

Use of metal stubs made the handling and treatment of the specimens much simpler. It also decreased the possibility of contamination. The use of the stub allowed for the distention of the lamina propria, so that the fishnet pattern can be observed readily. During freeze-drying, the use of metal stubs was extremely useful as the metal stub conducted heat away from the specimens.

B. Discussion and Evaluation of the Dehydration Technique

Initial use of the chemical dehydration process discussed in Chapter IV, was very labour intensive, slow and artifact forming. Development and perfection of the freeze-drying technique improved the quality of the specimens, shortened and simplified the procedures

necessary for the preparation of the specimens, and, more importantly, released the investigator from unnecessary technical and manual chores. The present freeze-drying device is very simple and functional. The sources of incidental contamination have been reduced to a minimum, though it may occur when transferring the sample from the treatment surface and fixation dish into the freeze-drier. However, the time interval, during which the sample is exposed to contamination, is negligible. The present technique has an acceptable lack of artifact formation due to low ice crystal formation and shrinkage during dehydration. The dehydration process itself is quite flexible, it is controllable and has a relatively high efficiency. Using this system, we may dry five cubic centimeters of specimen within 2-3 hours. The size of the ice crystal is being kept to a minimum. The freeze-drier platform can be cooled down to -150° if necessary. In addition, the tissues may be freeze-dried with 20-30% alcohol. The results are reproducible, and the micrographs are of high quality. The process is safe for handling and is quite reliable.

Use of isopentanol for initial freezing of the sample is very important. The isopentanol, when cooled down to -90°C, removes heat from the sample very rapidly without boiling off. The rapid temperature change reduces the formation of large ice crystals in the frozen tissue, thus reducing tissue damage and distortion. Maintaining the sample at very low temperatures and freeze-drying it with 20-30% alcohol will keep ice crystal formation to a minimum.

Discontinuation of use of osmium, on the surface of the sample did not adversely affect the images quality under SEM. It had been deposited on the surface of the specimen to decrease the electrostatic

charging.

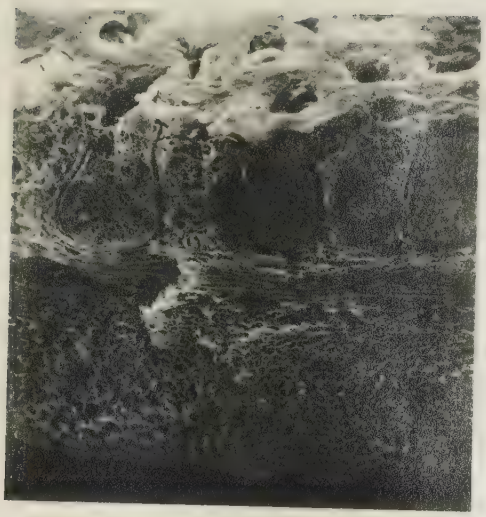
Use of freeze fracturing of the tissues under extremely low temperatures (-190°C or less) produced almost artifact-free surfaces of the cross-section of lamina propria and submucosa. Several examples of this are illustrated in Figure 33.

C. Distortions of the Lamina Propria and Submucosa Specimens During Freeze-Drying

It is very important to establish the degree of shrinkage and distortion produced by freeze-drying prior to observation of the surfaces of the tissue in the lamina propria, it was not very difficult. Using the scanning light microscope, scraped and stained lamina propria preparations were photographed under 100 x magnification (Figure 7). The dimensions of the lamina propria grid were measured from the photographs and compared to the SEM images photographed and measured at similar magnifications, with no noticeable differences. Another indirect way of judging distortion during freeze-drying, is by utilizing the cross-sections obtained by freeze fracturing (Figure 33). If there is a significant degree of shrinkage during the process of freezing and drying, the following would occur. In micrographs where the epithelial layer, the lamina propria and the basement membrane are seen, the gap between the basement membrane and the epithelial layer would be significant. This would occur for the following reasons. Significant shrinkage of the basement membrane and the lamina propria would increase the volume of the Crypt of Lieberkühn. On the other hand, the cup formed by the columnar epithelium would decrease in size, thus creating a gap between the lamina propria and the columnar cells. This was not the case in the specimens observed.

Fig. 33. Four SEM micrographs of three different cross sections of the colonic wall of the dog.

Magnifications: Plate A, 200x; Plate B, 1000x; Plate C, 100x; Plate D, 200x.



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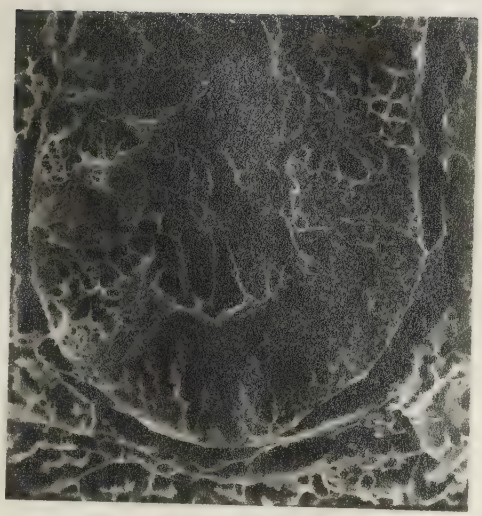

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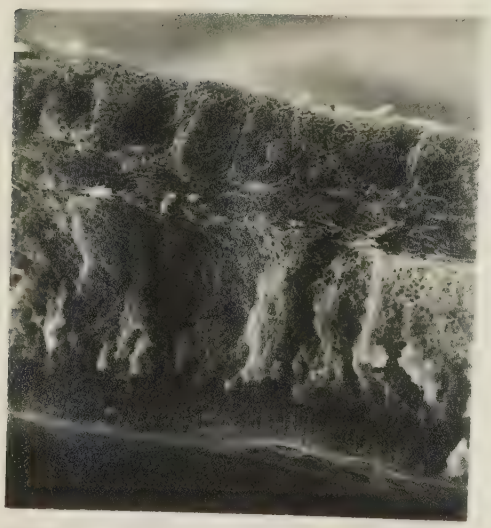

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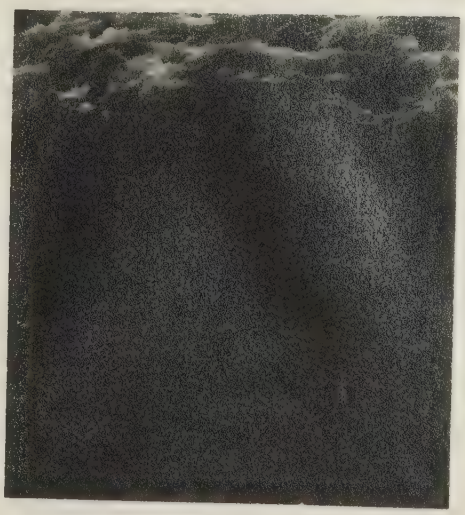

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D. Distortion Due to Ice Crystal Damage

Formation of large ice crystals during the freeze-drying may distort the cellular components and microstructure of the observed tissue. The presence of ice crystals in the tissue is indicated by disrupted membranes, haphazard distortion of recognizable structures, and appearance of large cavities in the cross-sections. The size of the ice crystals in the tissue is dependent on several factors such as rapidity of freezing, ability to maintain very low temperatures, the speed of ice sublimation and the ability to maintain a water-free state when the tissue is stored in subzero temperatures.

We have had little problem with ice crystal formation. The problem of conventional ice crystal formation has been addressed in the previous two sections.

E. Preservation of Surface Proportions

Demonstrating lamina propria, muscularis mucosae, submucosa, tunica muscularis and the mucosal coverings all in one frame enables the observer to compare the dimensions measured from these micrographs to those measured by other means such as light microscopy. Doing so, no significant difference in dimensions is noted. From the histological point of view, use of freeze fracturing provides a rather diagrammatic illustration of the structure of the bowel wall (Figure 33).

F. Safeguards Against Sample Contamination

1) Vacuum pump oil contamination. A great deal of grief was caused by contamination of the specimens with backstreaming light fractions of hot vacuum oil. An example of such a disaster is demon-

strated in Figure 34. To prevent such complications, the vacuum pumps had to be ballasted prior to use in order to clean the oil sufficiently. The pumps must be prevented from pumping significant amounts of water vapor by use of desiccant. Filters and a specially designed vacuum vessel for holding the liquid nitrogen are mandatory to condense and trap the oil that manages to ascend through the vacuum lines. The apparatus should be cleaned after 40-60 hours of continuous use.

2) Ambient air contamination. If the wet or dry sample is exposed to unfiltered room air for long, it may collect dust and other microscopic impurities from the air. Similarly, a leak in the vacuum seal not only makes maintenance of sufficiently high vacuum difficult, but it may introduce contaminant vapors from the air which tend to condense on the surface of the very cold specimens. Thus, not only minute particles are deposited, but also crystals and condensates from the room air settle on the specimen surface.

3) Impure solutions during preparation. Use of tap water, saline, or non-certified formalin may cause problems with deposition of unwanted salts and other crystals. Hypotonic, double-distilled water prevents salt and crystal contamination without distortion, as contact with the hypotonic solution is only 1-2 minutes.

By eliminating unnecessary steps in the preparation of the tissue, such as chemical dehydration, the probability of tissue contamination has been considerably diminished. In the present form, the dehydration technique uses only formalin and double-distilled water with little contaminant deposition.

Fig. 34. Examples of severe contamination.

A. Vacuum oil condensed on the surface of submucosa. Note tiny droplets.

B. Ambient air contamination.

A

B

4) Use of fresh desiccant at all times. Use of fresh phosphorus pentaoxide shortens the sublimation period, necessary for the dehydration of the specimens and permits a much higher vacuum. This compound will remain inert while it is dry, however, when hydrated this compound will lose its desiccant properties and evaporate phosphoric acid. The phosphoric acid will rapidly condense on the cold surfaces of the specimens and quickly destroy the samples. For best results, every cycle of dehydration should have a new desiccant.

G. Advantages and Disadvantages of Scanning Electron Microscopy

Use of SEM has produced a number of breathtaking views of the detailed ultrastructure of biologic matter (Figure 35). Some of the important advantages of SEM are: 1) three dimensional viewing of the specimens showing precise surface topography and structure; 2) it is relatively simple and it induces very little artifact; 3) the technique is very versatile, and can be used for minerals, metals, non-organic, organic and biological compounds; 4) it is commercially available; 5) minute specimens can be observed; 6) the equipment is easy to handle and is quite reliable in unprofessional hands, unlike the more sophisticated and sensitive transmission electron microscope; 7) the cost of SEM is justifiable in view of the vast amount of information available via this technique; and, 8) the speed with which the specimen may be prepared and observed.

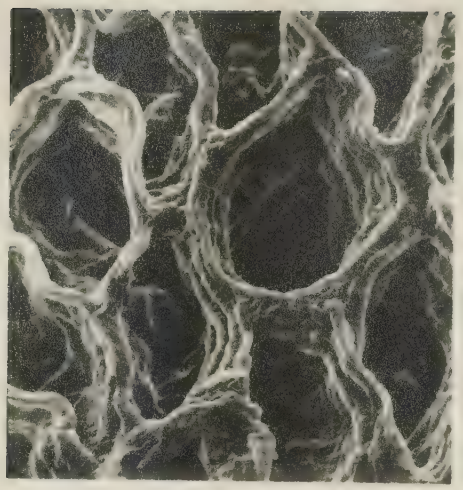
However, there are some disadvantages to using SEM: 1) the technique requires highly dehydrated specimens. However, recent developments have been achieved where the sample may be frozen without drying and can be placed on the low temperature observation stage, in

Fig. 35. SEM micrographs of lamina propria and submucosa. Note the degree of definition, lack of debris and remarkable depth of field.

A. Rat lamina propria

B. Human lamina propria

C. Canine submucosa



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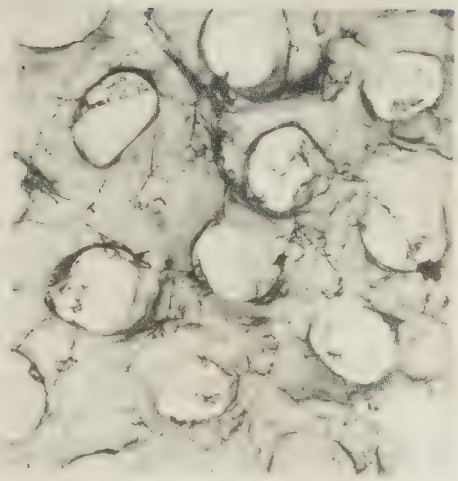

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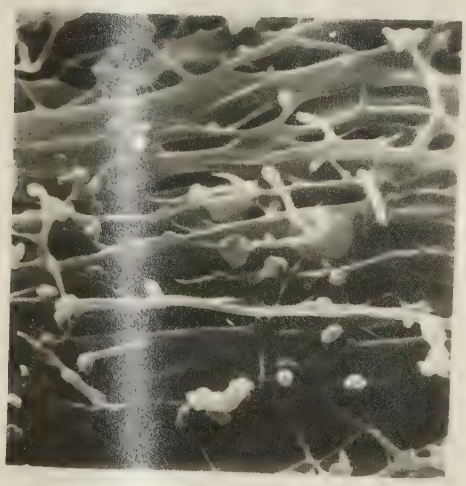

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in its natural state; 2) the maximum magnification of the best solution of SEM does not approach the resolutions achievable by the latest transmission electron microscopes. However, for most architectural and structural studies, as in our case, the SEM technique is adequate; 3) only structures on the surface and immediately beneath the surface can be observed; 4) possible thermal damage from high energy concentration of the electron scanning beam over a tiny area of observed surface at high magnification. This problem may soon be eliminated by the development of a low-energy electron gun.

In spite of these problems, SEM is an excellent tool for investigation of surface structure. As the SEM becomes more available and cheaper, it may result in acceptance of the scanning electron microscopic technique not only for biological and medical research, but also for possible clinical application, especially in the field of pathology.

CHAPTER IX

SUMMARY

SUMMARY

The objective of this work, as stated in the initial pages of this paper, was to develop the techniques necessary to observe normal lamina propria and submucosa of the gastrointestinal tract in at least three species. The results of this work are intended to be a platform, from which investigations into pathological states of colon will be made utilizing a relatively new tool, the scanning electron microscope. During the course of presentation of the study, the chronological development of surface preparation and dehydration techniques were presented.

The most recent technique of crushing of epithelial layer and debriding the lamina propria with a straight scalpel blade followed by the fixation, rapid freezing and dehydration of the specimen, produced micrographs of high quality. The technique is applicable to both the lamina propria and submucosa. Evidence was presented that the technique has negligible artifact-creating potential as long as the apparatus constructed is properly maintained.

During the course of the study, multiple regions of the intestinal tract were investigated with special emphasis to the colonic lamina propria and submucosal structure. Features such as trabeculation, fenestration, lowering of the cryptal walls, irregularity of the cryptal arrangement, were readily observed indicating that SEM may be a satisfactory tool for observation of the surface changes found in the diseased colon.

Most importantly, the normal lamina propria and submucosa in rat, dog, and man were described in terms of visual impact and measure-

characteristics.

The lamina propria in all three models was reminiscent of a fishnet arrangement. The Crypts of Lieberkühn, perforating the lamina propria, had ovoid or diamond shapes. There was very little difference in the dimensions of the crypts themselves between the three species. However, in the rat, the crypts were located very close to each other, with a resulting thin intercryptal wall. In the dog, the walls of the crypts were thicker and more regular. The human lamina propria had thick intercryptal walls with quite obvious perforations of basement membrane.

On the other hand, the submucosa was found to be arranged from three types of fibers (Chapter VI). These fibers form lamallae or layers of parallel-running fibers or fiber bundles, which interconnect with each other by thin, single collagen fibers. There appeared to be no significant difference between the collagen fiber arrangement in the three species. The human submucosal examples were of better quality, which may be due to gradual improvement of techniques. In the mass of submucosa, no vascular plexuses were observed and no cellular matter was noted.

A correlation between the structure and dynamic function, of both lamina propria and submucosa, was suggested.

The information included in this thesis may serve as a stepping stone for future work in determining the use of SEM for observation, description and diagnosis of disease states of the intestinal tract.

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